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(54) Title: COMPOUNDS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT AND PROPHYLAXIS OF BAC-TERIAL INFECTIONS

(57) Abstract

    Novel methods for the treatment and/or prophylaxis of diseases caused by tissue-adhering bacteria are disclosed. By interacting with periplasmic molecular chaperones it is achieved that the assembly of pili is prevented or inhibited and thereby the infectivity of the bacteria is diminished. Also disclosed are methods for screening for drugs as well as methods for the de novo design of such drugs, methods which rely on novel computer drug modelling methods involving an approximative calculation of binding free energy between macromolecules. Finally, novel pyranosides which are believed to be capable of interacting with periplasmic molecular chaperones are also disclosed.

COMPOUNDS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT AND PROPHYLAXIS OF BACTERIAL INFECTIONS

FIELD OF THE INVENTION

The present invention relates to methods for the treatment and/or prophylaxis of diseases caused by tissue-adhering
10 pilus-forming bacteria by interaction with the binding between pilus subunits and periplasmic chaperones. The invention further relates to methods for identifying/designing substances capable of interacting with periplasmic chaperones and methods for identifying binding sites in periplasmic
15 chaperones. Finally, the invention relates to novel substances capable of interacting with periplasmic chaperones as well as pharmaceutical preparations comprising substances capable of interacting with periplasmic chaperones.

BACKGROUND OF THE INVENTION

20 Pathogenic Gram-negative bacteria cause a number of pathological conditions such as bacteraemia, bacteria-related diarrhoea, meningitis and (very commonly) urinary tract infections, i.a. pyelonephritis, cystitis, urethritis etc.

Urinary tract infections are one of the major causes of
25 morbidity in females. Despite the overall importance of urinary tract infections in women, there have been few efforts to apply novel strategies in order to treat and/or prevent these diseases. Commonly, conventional antibiotics are used to treat these infections, such as treatment with
30 penicillins, cephalosporins, aminoglycosides, sulfonamides and tetracyclines; in the special case of urinary tract infections, urinary antiseptics such as nitrofurantoin and

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nalidixic acid are employed, too. However, emerging anti-
biotic resistance will in the future hamper the ability to
successfully treat urinary tract infections. Multiple anti-
biotic resistance among these uropathogens is increasing. It

5   has been estimated that the annual cost for evaluation and
treatment of women with urinary tract infections exceeds one
billion dollars. In addition, approximately one-fourth of the
yearly 4 billion dollar cost attributed to nosocomial infec-
tions is a consequence of urinary tract infections. Among the

10 causative agents of urinary tract infections, *Escherichia
coli* clearly predominates among Gram-negative bacteria.

Pathogenic gram negative bacteria, notably *Escherichia coli*,
Haemophilus influenzae, *Salmonella enteriditis*, *Salmonella
typhimurium*, *Bordetella pertussis*, *Yersinia pestis*, *Yersinia

15 enterocolitica Helicobacter pylori*, and *Klebsiella pneumoniae*
owe part of their infectability to their ability to adhere to
various epithelial tissues. Thus, e.g. *E. coli* adhere to the
epithelial cells in the upper urinary tract in spite of the
flushing effect of unidirectional flow of urine from the

20 kidneys.

As indicated above, the above mentioned bacteria are involved
in a variety of diseases: Urinary tract infections (*E. coli*),
acute diarrhoea (*E. coli*, *Y. enterocolitica* and *Salmonella
spp*), meningitis (*E. coli* and *H. influenzae*), whooping cough

25 (*B. pertussis*), plague (*Y. pestis*), pneumonia and other
respiratory tract infections (*K. pneumoniae*, *H. influenzae*)
and peptic ulcer (*H. pylori*).

The initiation and persistence of many bacterial infections
such as those described above is thought to require the

30 presentation of adhesins on the surface of the microbe in
accessible configurations which promote binding events that
dictate whether extracellular colonization, internalization
or other cellular responses will occur. Adhesins are often
components of the long, thin, filamentous, heteropolymeric

35 protein appendages known as pili, fimbriae, or fibrillae

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(these three terms will be used interchangeably herein). The
bacterial attachment event is often the result of a stereo-
chemical fit between an adhesin frequently located at the
pilus tip and specific receptor architectures on host cells,
5   often comprising carbohydrate structures in membrane associ-
ated glycoconjugates.

Uropathogenic strains of *E. coli* express P and type 1 pili
that bind to receptors present in uroepithelial cells. The
adhesin present at the tip of the P pilus, *PapG* (pilus asso-
10   ciated polypeptide G), binds to the Galα(1-4)Gal moiety
present in the globoseries of glycolipids, while the type 1
adhesin, *FimH*, binds D-mannose present in glycolipids and
glycoproteins. Adhesive P pili are virulence determinants
associated with pyelonephritic strains of *E. coli* whereas
15   type 1 pili appear to be more common in *E. coli* causing
cystitis. At least eleven genes are involved in the
biosynthesis and expression of functional P pili; the DNA
sequence of the entire pap gene cluster has been determined.
P pili are composite heteropolymeric fibres consisting of
20   flexible adhesive fibrillae joined end to end to pilus rods.
The pilus rod is composed of repeating *PapA* protein subunits
arranged in a right handed helical cylinder. Tip fibrillae
which extend from the distal ends of each pilus rod were
found to be composed mostly of repeating subunits of *PapE*
25   arranged in an open helical conformation. The *PapG* adhesin
was localized to the distal ends of the tip fibrillae, a
location which is assumed to maximize its ability to recog-
nize glycolipid receptors on eukaryotic cells. Two minor
pilus components, *PapF* and *PapK*, are specialized adaptor
30   proteins found in the tip fibrillum. *PapF* links the adhesin
moiety to the fibrillum while *PapK* joins the fibrillum to the
pilus rod. The composite architecture of the P pilus fibre
reveals the strategy used by uropathogenic *E. coli* to present
the *PapG* adhesin to eukaryotic receptors. The rigid *PapA* rod
35   extends the adhesin away from interference caused by LPS and
other components at the bacterial cell surface while the
flexible fibrillum allows *PapG* steric freedom to recognize

and bind to the digalactoside moiety on the uroepithelium.
With a few exceptions, the structural organization of type 1
pili is very similar to that described for P pili. In type 1
pili, the mannose binding fibrillar tip adhesin is known as
FimH.

The assembly of virulence-associated pili in Gram-negative
pathogens requires the function of periplasmic chaperones.
Molecular chaperones are vital components of all living
cells, prokaryotic and eukaryotic. Chaperones serve a variety
of cellular functions including folding, import and export of
proteins in various cellular compartments (Gething and Sam-
brook, 1992). Thus, a periplasmic chaperone is a molecular
chaperone which exerts its action in the periplasmic space in
bacteria.

PapD and FimC are the periplasmic chaperones that mediate the
assembly of P and type 1 pili, respectively. Detailed struc-
tural analyses have revealed that PapD is the prototype
member of a conserved family of periplasmic chaperones in
Gram-negative bacteria. These chaperones have a function
which is part of a general strategy used by bacteria to cap
and partition interactive subunits imported into the peri-
plasmic space into assembly competent complexes, making non-
productive interactions unfavourable. Determination of the
three-dimensional structure of PapD revealed that it consists
of two immunoglobulin-like domains oriented in a boomerang
shape such that a cleft is formed. PapD binds to each of the
pilus subunit types as they emerge from the cytoplasmic
membrane and escorts them in assembly-competent, native-like
conformations from the cytoplasmic membrane to outer membrane
assembly sites comprised of PapC. PapC has been termed a
molecular usher since it receives chaperone-subunit complexes
and incorporates, or ushers, the subunits from the chaperone
complex into the growing pilus in a defined order.

With the exception of the type IV class of pili, all other
genetically well characterized pilus systems in Gram-negative

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prokaryotes contain a gene analogous to PapD (Normark et al.,
1986; Hultgren et al. 1991); cf. also table A. FanE, faeE,
sfaE, ClpE and f17-D have been sequenced (Lintermans, 1990;
Schmoll et al., 1990; Bertin Y et al., 1993; Bakker et al.,
5   1991) and encode pilus chaperones required for the assembly
of K99, K88, S and F17 pili, respectively, in E. coli. The
assembly of Klebsiella pneumoniae type 3 pili and Haemophilus
influenzae type b pili requires the mrkb and hifB gene pro-
ducts, respectively (Gerlach et al., 1989,; Allen et al.,
10 1991). The structure-function relationships of all of these
chaperones have been analyzed using their amino acid
sequences and information from the crystal structure of PapD
(Anders Holmgren et al., 1992). The results have provided
insight into the molecular intricacies that have been evolu-
15 tionarily conserved in this class of proteins and suggested
significant structural similarities to immunoglobulins.

PapD is thus the prototype member of a family of periplasmic
chaperone proteins which are necessary for the correct
supramolocular assembly of bacterial pili. Chaperones such as
20 PapD in E. coli are required to bind the above-mentioned
pilus proteins imported into the periplasmic space, partition
them into assembly competent complexes and prevent non-pro-
ductive aggregation of the subunits in the periplasm (Dodson
et al., 1993).

25 In the absence of an interaction with the chaperone, pilus
subunits aggregate and are proteolytically degraded (Kuehn,
Normark, and Hultgren, 1991). It has recently been discovered
(Strauch, Johnson and Beckwith, 1989) that the DegP protease
is greatly responsible for the degradation of pilin subunits
30 in the absence of the chaperone. This discovery has allowed
the elucidation of the fate of pilus subunits expressed in
the presence or absence of the chaperone using monospecific
antisera in western blots of cytoplasmic membrane, outer
membrane and periplasmic proteins prepared according to
35 standard procedures. Expression of papG or papA in the degP41
strain (a DegP⁻ E. coli strain) in the absence of a chaperone

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was toxic to the bacteria due to the accumulation of these
proteins in the cytoplasmic membrane suggesting that the
chaperone was required for subunit import into the periplas-
mic space. The severity of the growth defect was related to
5   the level of expression of the pilin subunit, and was gen-
erally more dramatic with PapG than PapA. Co-expression of
PapD under the control of the inducible arabinose promoter
rescued the growth defect associated with subunit expression
in the degP41 strain and allowed PapG to be imported into the
10 periplasm.

Up to this time, little has elucidated about the molecular
recognition motifs of chaperones. Cytoplasmic chaperones such
as SecB, GroEL and DNaK bind to a diverse group of unfolded
target proteins in a sequence independent manner (Gething and
15 Sambrook, 1992). Recently, it has been suggested that DNaK
binds mainly via the target's peptide backbone (Landry et
al., 1992) and that GroEL may rely more on side-chain hydro-
phobicity and the ability of the target sequence to form an
amphipathic α-helix (Landry and Gierasch, 1991). PapD dif-
20 fers, however, in that it seems to bind its target proteins
in folded conformations (Kuehn et al., 1991).

The three-dimensional structure of PapD has previously been
solved (Holmgren and Bränden, 1989). This has shown PapD to
consist of two globular domains positioned such that the
25 overall shape of the molecule resembles a boomerang with a
cleft between the two domains. Each domain is a β-barrel
structure formed by two antiparallel β-pleated sheets, packed
tightly together to form a hydrophobic core, with a topology
similar to that of an immunoglobulin fold. The N-terminal
30 domain of PapD most resembles an Ig variable domain whilst
the C-terminal domain of PapD resembles CD4 (Wang et al.,
1990, Ryu et al., 1990) and the human growth hormone receptor
(de Vos et al., 1992).

A structural alignment between PapD and several periplasmic
35 chaperones predicted to have a similar immunoglobulin-like

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structure has identified invariant, highly conserved and
variable residues within this protein family (Holmgren et
al., 1992). Most conserved residues seem to participate in
maintaining the overall structure and orientation of the
5   domains towards one another. However, two conserved residues,
Arg-8 and Lys-112 are surface exposed and oriented towards
the cleft between the domains. Site-directed mutagenesis of
the Arg-8 amino acid has shown that it form at least part of
the pilus subunit binding pocket (Holmgren et al., 1992;
10   Kuehn et al., 1993).

From a sequence analysis of a number of the above-mentioned
pilus subunit proteins, it has been observed that they pos-
sess a number of common features including homologies at the
C termini (see also example 2). It is thought that these
15   similarities in sequence may be responsible for some function
common to all the pilus proteins, such as binding to their
periplasmic chaperone. Indeed, the C-terminal region of the
P-pili adhesin PapG has already been shown to be important in
the in vivo binding to PapD (Hultgren et al., 1989). Table A
20   lists 16 periplasmic proteins, all involved in assembly of
cell surface structures in pathogenic bacteria and all with
significant homology with PapD.

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Table A

Organism	Chaperone	Structure Assembled	Reference
E. coli	PapD	P pili	Lund et al., 1987
E. coli*	FimC	Type 1 pili	Klemm et al., 1992
E. coli	SfaE	S pili	Schmoll et al., 1990
E. coli	FaeE	K88 pili	Bakker et al., 1991
E. coli	FanE	K99 pili	Bakker et al., 1991
E. coli	CS3-1	CS3 pili	Jalajakumari et al., 1989
E. coli	F17D	F17 pili	Lintermans et al., 1990
E. coli	ClpE	CS31 A	Bertin et al., 1993
E. coli	EcpD	not identified	Raina et al., 1993
E. coli	CssC	Antigen CS6	
E. coli	NfaE	Nonfimbrial adhesin 1	
E. coli	AggD	Aggregative Adherence Fimbria 1	
K. pneumoniae	MrkB	Type 3 pili	Allen et al., 1991
B. pertussis	FimB	Type 2 & 3 pili	Locht et al., 1992; Willems et al. 1992
S. enteriditis	SefB	unknown pili	Clouthier et al., 1993
S. typhimurium	PefD	PEF	
H. influenzae	HifB	unknown pili	Smith et al., 1993
Y. enterocolitica	MyfB	Myf fibrillae	Iriarte et al., 1993
Y. pestis	PsaB	pH6 antigen	Lindler et al., 1992
Y. pestis	CaflM	F1 envelope antigen	Galyov et al., 1991
P. mirabilis	MrpD	MR/P Fimbriae	
?	YehC	?	

* This chaperone is present in all Enterobacteriaceae, since all members
of this family produce type 1 pili.

In summary, the three-dimensional structure of PapD as well
as the function of PapD and other periplasmic chaperones are
known, whereas the exact motif of binding between PapD and
the pilus subunits has been unknown until now.

DISCLOSURE OF THE INVENTION

According to the present invention, it has been realized that
the above-mentioned characteristics of PapD and other related

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chaperones make them interesting targets for drugs designed
primarily to reduce the pathogenicity of bacteria which
adhere by means of pili; for example, a drug which blocks the
binding between a chaperone and the pilus-subunits (thereby
5   interfering with the assembly of the intact pilus) will
interfere with the formation of intact pili, thereby reducing
bacterial capacity to adhere to host epithelium.

In order to design such a drug it is of great value that the
motif of binding between the chaperone and the pilus pro-
10 tein(s) is known in detail, in order to develop the method to
effectively identify compounds capable of blocking this
binding.

An aspect of the current invention relates to a method for
the treatment and/or prophylaxis of diseases caused by tis-
15 sue-adhering pilus-forming bacteria, comprising preventing,
inhibiting or enhancing binding between at least one type of
pilus subunit and at least one type of molecular chaperone in
the pilus-forming bacteria, which molecular chaperone binds
pilus subunits during transport of these pilus subunits
20 through the periplasmic space and/or during the process of
assembly of the intact pilus.

As used herein, the term "pilus", "fimbria", or "fibrilla",
relates to fibrillar heteropolymeric structures embedded in
the outer membrane of many tissue-adhering pathogenic
25 bacteria, notably the pathogenic gram negative bacteria. In
the present specification the terms pilus, fibrillum and
fimbria will be used interchangeably. A pilus is, as
explained above, composed of a number of "pilus subunits",
which constitute distinct functional parts of the intact
30 pilus. A very important pilus subunit is the "adhesin", the
pilus subunit which is responsible for the tissue-binding
capacity of the bacterium.

By the term "molecular chaperone" is meant a molecule which
in living cells has the responsibility of binding to peptides

in order to mature the peptides in a number of ways. Many
molecular chaperones are involved in the process of folding
of peptides into their native conformation whereas other
molecular chaperones are involved in the process of export
5 out of or import into the cell of peptides. Specialized
molecular chaperones are "periplasmic chaperones", which are
bacterial molecular chaperones exerting their main actions in
the "periplasmic space" (the space between the inner and
outer bacterial membrane). Periplasmic chaperones are
10 involved in the process of correct assembly of intact pili.
When used herein, the simple term "chaperone" designates a
molecular, periplasmic chaperone if nothing else is indi-
cated.

When using the phrase "one type of" is meant that the pilus
15 subunit or the chaperone in question is of one distinct
species. However, especially the fact that there is extensive
homology between different species of periplasmic molecular
chaperones renders it likely that the interference with one
type of chaperone using e.g. a compound will make it possible
20 to also use the compound in interference with other chape-
rones.

The phrase "preventing, inhibiting or enhancing binding
between pilus subunits and at least one molecular chaperone
in the pilus-forming bacteria" indicates that the normal
25 interaction between a chaperone and its natural ligand, i.e.
the pilus subunit, is being affected either by being com-
pletely or substantially completely prevented, or by being
inhibited, or expressed in another manner, reduced to a such
an extent that the binding of pilus subunits to the chaperone
30 is measurably lower than is the case when the chaperone is
interacting with the pilus subunit at conditions which are
substantially identical (with regard to pH, concentration of
ions and other molecules) to the native conditions in the
periplasmic space. Similarly, the enhancement of binding
35 between the chaperone and the pilus subunit should be such
that the binding of pilus subunits to the chaperone is measu-

rably higher than is the case when the chaperone is interacting with the pilus subunit at conditions which are substantially identical (with regard to pH, concentration of ions and other molecules) to the native conditions in the periplasmic space. Measurement of the degree of binding can be determined *in vitro* by methods known to the person skilled in the art (microcalorimetri, radioimmunoassays, enzyme based immuno assays, etc).

It should, on the basis of the above, be clear that prevention or inhibition of the normal interaction between a pilus subunit and a chaperone should have a substantially limiting effect on pilus assembly. However, an enhancement of the binding between pilus subunits and chaperones may also prove to be devastating to a bacterium. As will appear from example 2, different pilus subunits bind to PapD with different affinities and affecting this narrowly balanced system may also cause a limitation on the rate and efficiency of pilus assembly.

It is believed that even modest changes in the binding between pilus subunits and chaperones can have dramatic impact on the efficiency of pilus assembly, and thus on the ability of the bacteria to adhere. For example, if the change in the binding between a chaperone and one pilus subunit is such that the normal order of affinities between the chaperones and the pilus subunits which normally bind thereto is altered, then the normal assembly of the pilus should be disturbed, since the order of assembly of the pilus may be dependent i.a. on the affinities between the pilus subunits and the chaperone: The pilus subunits with the highest affinities to the chaperone may be incorporated before other pilus subunits with lesser affinities.

Thus, prevention, inhibition or enhancement of binding between pilus subunits and a periplasmic molecular chaperone have the effect of impairing pilus assembly, whereby the

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infectivity of the microorganism normally expressing the pili is reduced.

Prevention, inhibition or enhancement of the binding between pilus subunits can be accomplished in a number of ways. A
5   preferred method according to the invention of treatment and/or prophylaxis of diseases caused by tissue-adhering pilus-forming bacteria is to administer an effective amount of a substance to a subject in need thereof, the substance being capable of interacting with at least one type of mole-
10 cular chaperone which binds pilus subunits during transport of these pilus subunits through the periplasmic space and/or during the process of assembly of the intact pilus, in such a manner that binding of pilus subunits to the molecular chaperone is prevented, inhibited or enhanced.

15 The substance can be any compound which has one of the above mentioned effects on the interaction between chaperones and pilus subunits and thereby on the assembly of the pilus. Especially interesting substances are those which are likely to interact with the pilus subunit binding part of the chape-
20 rone, but interaction with other sites in the chaperones may also cause prevention, inhibition or enhancement of the binding between pilus subunits and the chaperone. This can be an effect of direct steric blocking of the normal binding between the subunit and the chaperone, but it may also be an
25 effect of a conformational change in the chaperone. A method of identifying substances to be used in the method of the invention is disclosed below.

The interaction between the substance and the chaperone may be a covalent as well as a non-covalent binding to the chape-
30 rone by the substance.

By the term "subject in need thereof" is in the present context meant a subject, which can be any animal, including a human being, who is infected with, or is likely to be

infected with, tissue-adhering pilus-forming bacteria which
are believed to be pathogenic.

By the term "an effective amount" is meant an amount of the
substance in question which will in a majority of patients
5   have either the effect that the disease caused by the
pathogenic bacteria is cured or, if the substance has been
given prophylactically, the effect that the disease is pre-
vented from manifesting itself. The term "an effective
amount" also implies that the substance is given in an amount
10   which only causes mild or no adverse effects in the subject
to whom it has been administered, or that the adverse effects
may be tolerated from a medical and pharmaceutical point of
view in the light of the severity of the disease for which
the substance has been given.

15   The route of administration of the substance could be any
conventional route of administration, i.e. oral, intravene-
ous, intramuscular, intradermal, subcutaneous etc. The oral
route is preferred.

The dosage of such a substance is expected to be the dosage
20   which is normally employed when administering antibacterial
drugs to patients or animals, i.e. 1 μg - 1000 μg per kilo-
gram of body weight per day. The dosage will depend partly on
the route of administration of the substance. If the oral
route is employed, the absorbtion of the substance will be an
25   important factor. A low absorbtion will have the effect that
in the gastro-intestinal tract higher concentrations, and
thus higher dosages, will be necessary. Also, the dosage of
such a substance when treating infections of the central
nervous system (CNS) will be dependent on the permeability of
30   the blood-brain barrier for the substance. As is well-known
in the treatment of bacterial meningitis with penicillin,
very high dosages are necessary in order to obtain effective
concentrations in the CNS.

It will be understood that the appropriate dosage of the
substance should be suitably be assessed by performing animal
model tests, wherein the effective dose level (e.g. ED_{50}) and
the toxic dose level (e.g. TD_{50}) as well as the lethal dose
5 level (e.g. LD_{50} or LD_{10}) are established in suitable and
acceptable animal models. Further, if a substance has proven
efficient in such animal tests, controlled clinical trials
should be performed. Needless to state that such clinical
trials should be performed according to the standards of Good
10 Clinical Practice.

Although the preferred way of preventing, inhibiting or
enhancing the binding between pilus subunits and chaperones
is to administer a substance with the above mentioned effects
on the chaperone, other ways are possible. For instance,
15 substances interacting with one type of pilus subunit could
also have the effects described above, and for the same
reasons. However, as the interaction with the chaperone is
likely to exert effects on the assembly into the pilus of
most, if not all, pilus subunits constituting the intact
20 pilus, it is expected that the interaction with the chaperone
will be the most efficient in terms of hampering bacterial
infectivity.

As will appear from the examples below, most of the data on
binding between chaperones and pilus subunits have been
25 obtained by studying the interaction between the *PapD* chape-
rone from *E. coli*. However, since many tissue adhering
bacteria have been found to express pili which share substan-
tial homologies in their C-terminal part, and since substan-
tial homologies have been demonstrated between the various
30 periplasmic chaperones which until now have been isolated
(see table A), it is justified to assume that some substances
and classes of substances will be capable of interacting with
the majority of existing periplasmic chaperones and thus be
useful in the treatment and/or prophylaxis of diseases caused
35 by the bacteria harbouring these chaperones when the sub-

stance is administered to patients infected with the
bacteria.

Thus, the method of the invention for the treatment and/or
prophylaxis is especially intended to be used in patients
5   which are infected by bacteria selected from the group con-
sisting of *Haemophilus* spp, *Helicobacter* spp, *Pseudomonas
aeruginosa*, *Mycoplasma spp*, and all members of the *Enterobac-
teriacieae* family, including *Escherichia* spp, *Salmonella* spp,
Bordetella spp, *Yersinia* spp, *Proteus* spp and *Klebsiella* spp.
10 In this connection, especially the bacteria selected from the
group consisting of *E. coli*, *Y. pestis*, *Y. enterocolitica*, *B.
pertussis*, *K. pneumoniae*, *S. typhimurium*, *S. typhi*, *S. para-
typhii*, *Helicobacter pylori*, *Proteus mirabilis* and *Haemophi-
lus influenzae* are regarded as infectants which cause infec-
15 tions which can be treated and/prevented by the use of the
method according to the invention.

Accordingly, in important aspects of the invention, the
binding of a pilus subunit to a chaperone selected from the
group consisting of *PapD*, *FimC*, *SfaE*, *FaeE*, *FanE*, *Cs3-1*,
20 *F17D*, *ClpE*, *EcpD*, *Mrkb*, *FimB*, *SefB*, *HifB*, *MyfB*, *PsaB*, *PefD*,
YehC, *MrpD*, *CssC*, *NfaE*, *AggD*, and *Caf1M* is prevented,
inhibited or enhanced. It is especially preferred that the
binding of *PapD* to at least one pilus subunit is affected.

As stated above, in a preferred embodiment of the invention,
25 the prevention, inhibition or enhancement of the binding is
accomplished by interacting with, in the molecular chaperone,
a binding site which is normally involved in binding to pilus
subunits during transport of these pilus subunits through the
periplasmic space and/or during the process of pilus assem-
30 bly.

As mentioned, in connection with the present invention, the
binding motif between *PapD* and a peptide which constitute the
19 amino acids of the C-terminal of *PapG* (G1'-19'), a pilus
subunit, has been determined. As is described in detail

herein, other chaperones share substantial homologies with
PapD in this binding site. Thus, such a binding site is of
great interest as a target for drugs which are intended to
interact with periplasmic chaperones. Therefore, in a pre-
5 ferred embodiment of the above described methods of the
invention, the binding site which is affected is one which
binds G1'-19'.

Thus, an important aspect of the invention is a method as
described above, wherein the binding site is a binding site
10 to which the carboxyl terminal part of a pilus subunit is
capable of binding, and which comprises site points substan-
tially identical to the invariant residues Arg-8 and Lys-112
in PapD, and a polypeptide fragment which is capable of
interacting with a β-strand of the carboxyl terminal part of
15 the pilus subunit thereby stabilizing the binding of said
subunit at the Arg-8 and Lys-112 site points of the binding
site. An especially preferred aspect of the invention is a
method as described above, where the binding side is the G-
protein binding site of PapD as described herein.

20 The term "site-point" refers to a chemical group with well
defined physical/chemical characteristics such as size,
charge, hydrophobicity/hydrophilicity, polarity, direction of
hydrogen bonds as well as a 3-dimensional position (distance
and angle) relative to other such chemical groups. Thus,
25 site-points which are "substantially identical" to Arg-8 and
Lys-112, are chemical groups which substantially share the
same well-defined physical/chemical characteristics as these
two amino acids.

The term "invariant residues" refer to amino acid residues
30 which can be found in a number of proteins without there
being any variation with regard to the precise type of the
amino acid and without there being any substantial variation
in their function in the proteins. The presence of invariant
residues in a large number of related proteins normally is an
35 indication of the biological importance of such residues,

since mutations lacking these residues apparently lacks the function of the intact protein, too. As described herein, it has been found that all periplasmic molecular chaperones share the amino acid residues which are equivalent to Arg-8 and Lys-112. It is believed that these two residues therefore are of considerable importance to pilus-forming bacteria.

"A polypeptide fragment capable of interacting with a β-strand of the carboxyl terminal part of a pilus subunit" indicates that part of the chaperone (which is also a part of the binding site) is capable of interacting with a β-strand of the pilus subunit. This interaction serves as a stabilizing factor in the binding between the pilus subunit and the chaperone and is considered a very important part of the total motif of binding between the chaperone and the pilus subunit. Further, it has recently been rendered probable by the inventors that the β-strand serves as a template for the correct folding of the pilus subunit (cf. example 10).

As explained herein, the C-terminal part of many, if not all, known pilus subunits, share substantial homologies, which is another indication of the importance of the 3-dimensional structure of the pilus subunit as well as of the chaperone in order for the binding to take place and be stable.

As appears from the examples, another binding site residing in domain 2 of PapD has been identified. This binding site interacts with fusion protein MBP-G1'-140' as well as with a short peptide constituted of the C-terminal amino acid residues 125' to 140' of PapG. Thus, also this binding site is of great interest as a target for drugs which are intended to interact with periplasmic chaperones. Therefore, a preferred embodiment of the above described methods of the invention is a method wherein the binding site which is affected is one which binds either of the two above-described peptides.

It will be understood that the above-described methods comprising administration of substances in treating and/or

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preventing diseases are dependent on the identification or de
novo design of substances which are capable of exerting
effects which will lead to prevention, inhibition or enhance-
ment of the interaction between pilus subunits and periplas-
5 mic molecular chaperones. It is further important that these
substances will have a high chance of being therapeutically
active.

Thus, an aspect of the invention relates to a method for
identifying a potentially therapeutically useful substance
10 capable of interacting with a periplasmic molecular chapero-
ne, thereby preventing, inhibiting or enhancing the interac-
tion between a periplasmic molecular chaperone and a pilus
subunit, the method comprising at least one of the following
steps:

15 1) testing a candidate substance in an assay in which the
possible prevention, inhibition or enhancement by the sub-
stance of the interaction between the periplasmic molecular
chaperone and the pilus subunit is determined by

a) adding the substance to a system comprising the periplas-
20   mic molecular chaperone or an analogue thereof in an
immobilized form and the pilus subunit or an equivalent
thereof in a solubilized form and determining the change
in binding between the pilus subunit or equivalent there-
of and the periplasmic molecular chaperone or analogue
25   thereof caused by the addition of the substance, or

b) adding the substance to a system comprising the pilus
subunit or an equivalent thereof in an immobilized form
and the periplasmic molecular chaperone or an analogue
thereof in a solubilized form and determining the change
30   in binding between the pilus subunit or equivalent there-
of and the periplasmic molecular chaperone or analogue
thereof caused by the addition of the substance, or

c)   adding the substance to a system comprising the pilus
     subunit or an equivalent thereof as well as the periplas-
     mic molecular chaperone or an analogue thereof in
     solubilized form and determining the change in binding
5   between the pilus subunit or equivalent thereof and the
     periplasmic molecular chaperone or analogue thereof
     caused by the addition of the substance, or

d)   adding the substance to a system comprising the pilus
     subunit or an equivalent thereof as well as the periplas-
10   mic molecular chaperone or an analogue thereof in
     solubilized form and measuring the change in binding
     energy caused by the addition of the substance, and
     identifying the substance as potentially therapeutically
     useful if a significant change in the binding energy
15   between the pilus subunit or equivalent thereof and the
     periplasmic molecular chaperone or analogue thereof is
     observed,

and identifying the substance as potentially therapeutically
useful if a significant change in the binding or binding
20 energy between the pilus subunit or equivalent thereof and
the periplasmic molecular chaperone or analogue thereof is
observed;

2)   testing a candidate substance in an assay in which the
possible prevention, inhibition or enhancement of the inter-
25 action between the periplasmic molecular chaperone and the
pilus subunit is determined by

adding the substance to a system comprising living tis-
sue-adhering pilus-forming bacteria followed by determi-
nation of the growth rate of the bacteria, a reduction in
30   growth rate compared to a corresponding system wherein
the substance has not been added being indicative of
prevention, inhibition or enhancement of the binding
between the periplasmic molecular chaperone and the pilus
subunit, or

adding the substance to a system comprising living tis-
sue-adhering pilus-forming bacteria followed by a deter-
mination of the tissue adhesion of the bacteria, a reduc-
tion in tissue adhesion compared to a corresponding

5 system wherein the substance has not been added being
indicative of prevention, inhibition or enhancement of
the binding between the periplasmic molecular chaperone
and the pilus subunit,

and identifying the substance as potentially therapeutically
10 useful if a reduction in growth rate or tissue adhesion is
observed after the addition of the substance; and

3)   administering, to an experimental animal, a sub-
stance which has been established in vitro to
prevent, inhibit or enhance the interaction
15   between a periplasmic molecular chaperone and a
pilus subunit, the experimental animal being ino-
culated with tissue-adhering pilus-forming
bacteria before, simultaneously with or after the
administration of the substance, and electing as a
20   substance suitably capable of interacting with a
periplasmic molecular chaperone, a substance pre-
venting and/or curing and/or alleviate disease
caused by the bacteria.

By the term "an equivalent of a pilus subunit" is meant a
25 compound which has been established to bind to the chaperone
in a manner which is comparable to the way the pilus subunit
binds to the chaperone, e.g. by the demonstration of the
pilus subunit and the equivalent competing for the binding to
the chaperone. Preferred equivalents of pilus subunits are
30 G1'-19'WT, MBP-G1'-140' and G125'-140', which are all
described in detail herein.

The term "an analogue of a chaperone" denotes any substance
which have the ability of binding at least one pilus subunit

in a manner which corresponds to the binding of said chape-
rone to a pilus subunit. Such an analogue of the chaperone
can be a truncated form of the intact chaperone (e.g. one of
the two domains of *PapD*) or it can be a modified form of the
chaperone which may e.g. be coupled to a probe, marker or
another moiety. Finally, the analogue of the of the chaperone
can be an isolated, but partially of fully functional, bin-
ding site of the chaperone or a synthetic substance which
mimics such a binding site.

The immobilisation mentioned above may be simple non-covalent
binding to an adhering surface or a host or receptor molecule
such as an antibody, or covalent binding to a spacer molecule
such as a polymer or a peptide.

In the above mentioned step 1a), the pilus subunit or the
equivalent thereof being bound to the periplasmic molecular
chaperone or an analogue thereof can be detected in a number
of ways, e.g. by the pilus subunit or the equivalent thereof
being labelled, or by means of a labelled ligand (such as an
antibody) capable of reacting with the pilus subunit or the
equivalent thereof, or by means of a refractive index based
determination of the extend of binding, such as the Pharmacia
BiaCore® assay.

Accordingly, in step 1b) the periplasmic molecular chaperone
or the analogue thereof being bound to the pilus subunit or
the equivalent thereof may be detected by the periplasmic
molecular chaperone or the analogue thereof being labelled,
by means of a labelled ligand (e.g. antibody) capable of
reacting with the periplasmic molecular chaperone or the
analogue thereof, or by means of a refractive index based
determination of the extend of binding, such as the Pharmacia
BiaCore® assay.

In step 1c) the periplasmic molecular chaperone or the ana-
logue thereof being bound to the pilus subunit or the equi-
valent thereof may be detected by separation of pilus sub-

unit/chaperon complexes (e.g. by ultracentrifugation,
ultrafiltration, liquid chromatography, such as size ex-
clusion chromatography, or electrophoresis). Described below
is a method relying on the changes in fluorescence of a short
5   PapG fragment when this fragment is bound to PapD. This
method is a preferred assay in the method of the invention.

The determination of binding energy in step 1c) is preferably
performed in a microcalorimetric system using the well-known
technique of microcalorimetry.

10 The above-indicated steps serve 3 purposes. The types of
assays in step 1) are intended to shed light over the ability
of the candidate substance of interacting with the chaperone.
In the instances wherein labelled substances, chaperones or
antibodies are used, the label could be a radioactive label,
15 a fluorescent or light absorbing label, an enzyme such as
horse-radish peroxidase, a ligand such as biotin, or any
other conventional labelling system known to the person
skilled in the art. The detection of the labelled compound is
then dependent on the choice of label: radioactivity may be
20 measured in a liquid-scintillation counter, a gamma counter,
or any other convenient detection system for radioactivity,
enzyme-labels are detected by the presence or absence of a
specific substrate for the enzyme (optical density assess-
ment, chemical reactivity of the remaining substrate or of
25 the product etc.), fluorescent labels may be detected by
fluorescence microscopy or simple measurement of the fluores-
cent emission, light-absorbing labels may be detected by
measurement of absorbtion of light of a characteristic wave-
length, and biotin may be detected by its binding to strept-
30 avidin.

The separation of high molecular complexes by ultracentri-
fugation or ultrafiltration in 1) may be detected by one of
the components of the complex being labelled as described
above; it is thus possible to detect the ratio between bound
35 and unbound pilus subunit/equivalent, but the detection step

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may also rely on the binding of antibodies to one of the
components of the complex, and the subsequent detection of
this antibody. Any conventional chromatographic technique may
be employed (HPLC, FPLC, size exclusion, etc) The separation
5   by electrophoresis may e.g. be performed by capillary elec-
trophoresis.

The assays in step 2) all relate to the effects of the candi-
date substance on bacterial activity *in vitro*. The demonstra-
tion of a reduction in growth rate of the bacteria or a
10 demonstration of reduced adherence to cells or synthetic
surfaces in an assay of course cannot be contributed to the
effect of interaction with chaperones only, but a demonstra-
tion of this kind should provide a good estimate of the
potential therapeutical usefulness of such a substance.

15 The determination of growth rate may be performed by counting
of colonies on solid agar plates striped with the bacteria,
by counting bacterial density in liquid growth media (OD$_{600}$
determination), by measuring fluorescence of substances such
as NAD(P)H, ATP, or amino acids, which are contained in the
20 bacterial cells only, or by any other convenient detection
system known to the person skilled in the art. The determina-
tion of adherence of the bacteria may be performed in a
similar manner after the adhering bacteria have been iso-
lated. A determination of adherence is preferably performed
25 by measuring the ability of the bacteria to agglutinate red
blood cells or receptor-coated latex beads, by measuring the
bacterial adhesion to receptor-coated microtiter plates, or
by measuring the bacterial adhesion to other synthetic sur-
faces.

30 In a preferred embodiment of the method described above, the
living, tissue adhering pilus-forming bacteria are of a
protease deficient strain, the protease being one which is at
least partially responsible for the degradation of pilus
subunits. One especially preferred type of strain is the
35 degP41 strain of *E. coli*. As described herein, the *degP41*

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strain lacks activity of the DegP protease which is responsible for degradation of pilus subunits in the *E. coli* when these are not rescued into periplasmic space by *PapD*, and *degP41* strains are thus especially sensitive to changes in
5   the efficiency of *PapD*, as the accumulation of pilus subunits is toxic to the cell. It is believed that equivalent proteases exist in other pilus expressing bacteria.

The animal study in step 3) is performed in order to demonstrate the potential therapeutic usefulness of the candidate
10 substance *in vivo*. Further, as already mentioned above, such animal studies should also establish the a *priori* values regarding effective dosage and toxicity before the candidate substance finally is tested in human beings in controlled clinical trials. The animal studies should also provide
15 information regarding the convenient formulation of the substance in a pharmaceutical preparation as well as the preferred route of administration, as it is possible to obtain, from the animal model, data for absorbtion of the substance as well as data for the metabolism and excretion of
20 the substance. The experimental animal is preferably a mouse, a rat, a cat, a dog, a monkey, a horse, a cow, a pig, or a chicken.

The term "suitably capable of interacting with a molecular chaperone" is intended to indicate that a substance, apart
25 from being capable of interacting with a molecular chaperone, also is capable of exerting effects in an *in vivo* system, i.e. that the substance in addition to its binding capability also exhibits compatibility with a biological system, i.a. a patient.

30 Although the above-indicated *in vivo* studies, especially the experiments in animal models, are the best indicators of the potential therapeutical usefulness of a substance in the prevention, inhibition, or enhancement of the binding between a chaperone and a pilus subunit, it should not be forgotten
35 that the *in vitro* assays outlined above serve as important

leads when developing compounds with a therapeutical poten-
tial. If one relied only on in vivo assays, it is very likely
that compounds which in fact exhibit the desired effect on
the chaperone/pilus subunit interaction would be screened out
5 by the in vivo assays, because these compounds could lack
e.g. the ability to penetrate biological membranes. When
using the in vitro assays, a much greater chance of finding a
lead compound is maintained.

The evaluation of the effect of a substance tested in the in
10 vitro assays described herein (cf. in this connection especi-
ally the examples) depends on a number of factors. It will be
understood by the skilled person that a small molecule could
be added in rather high molar concentrations in order to
exert an effect on the chaperone/pilus subunit interaction
15 (and even then the small molecule may still be an interesting
lead compound), whereas larger molecules may exert marked
effects even in rather low molar concentrations. In general,
when any in vitro assay described herein is regarded as
having a positive result when testing a candidate substance
20 (i.e. that the substance tested shows a "significant"
effect), the following condition should be fulfilled: The
compound should exert a significant effect on pilus sub-
unit/chaperone interaction (or on an interaction in an equi-
valent system which correlates well to pilus sub-
25 unit/chaperone interaction), the significant effect being one
which with no doubt can be attributed to the interaction
between the substance and the chaperone and which is not an
unspecific interaction between the chaperone and the sub-
stance (due to e.g. radical changes in the physical and
30 chemical environment when the substance is added). One way of
excluding unspecific interactions as the reason for the
exerted effect is to use at least one control which is a
chemically comparable substance (with respect to molecular
mass, charge/polarity and gross 3-dimensional conformation
35 (globular, fibrillar etc.). If the control does not result in
substantially the same effect in the assay as the substance,

it can be concluded that the substance must be regarded as an assay positive substance.

The assays described in the examples are all good examples of assay types, which could serve as the test system in the
5 above-described method of the invention. However, it is preferred that the method described in example 10 employing a fluorescence labelled variant of a pilus subunit is used in step 1c). This assay may shortly be described as follows:

- adding the substance to a first system comprising the
10 periplasmic molecular chaperone or an analogue thereof,

- subsequently adding a pilus subunit or an equivalent thereof which has been labelled with an environmentally sensitive fluorescent probe,

- determining the fluorescent emission at a particular wave-
15 length which is indicative of the amount of binding between the periplasmic molecular chaperone or the analogue thereof and the pilus subunit or the equivalent thereof, and

- comparing the determined fluorescent emission to fluorescent emission determined in a corresponding second system
20 containing substantially the same concentrations of the molecular chaperone or the analogue thereof and the pilus subunit or the equivalent thereof but substantially no substance,

a significant difference in fluorescent emission between the
25 first and second system being indicative of interaction between the periplasmic molecular chaperone or the analogue thereof and the substance.

The advantage of this assay is that it may be employed for quantitative determinations of the effect of the tested
30 substance on the chaperone/pilus subunit system. By using this assay the inventors have e.g. determined the constant of

binding between a PapG analogue and PapD. The quantitative
determinations may be performed by performing the determina-
tion of fluorescent emission in the second system a plurality
of times at varying molar ratios between the pilus subunit or
5   the equivalent thereof and the periplasmic chaperone and the
equivalent thereof, whereupon the constant of binding between
the pilus subunit or equivalent thereof and the periplasmic
molecular chaperone or analogue thereof is assessed from the
determined fluorescent emission data. From the data obtained
10   in this way it is also possible to determine the binding
constant of the substance in a parallel manner, which will
appear from claim 11.

It will be understood that the above-indicated method for
identifying a potentially therapeutically useful substance is
15   dependent on the actual presence of the substance. Normally,
it is necessary to either purify or synthesize the candidate
substance before it is subjected to the above-mentioned
method. However, since many such candidate substances are
likely to be tested before a substance which is suitably
20   capable of interacting with a chaperone will be identified,
it is of interest to identify such substances before they are
subjected to the method above, thereby diminishing the
resources spent on purification and/or synthesis steps.

Hence, the invention also relates to a method for identifying
25   and/or designing a substance, X, capable of interacting with
a chaperone, e.g. binding to the chaperone, with a predicted
binding energy equal to or better than a predetermined thres-
hold value, the method comprising

1)   selecting a substance, A, which could potentially inter-
30   act with a site in the chaperone, and providing a 3-
dimensional structural representation thereof,

2)   predicting the binding free energy between the substance
A and the site in the chaperone,

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3)   if the predicted binding free energy between the sub-
      stance A and the site in the chaperone is equal to or
      better than the predetermined threshold value, then
      identifying the substance A as the substance X,

5 4)   if the predicted binding free energy between the sub-
      stance A and the site in the chaperone is not equal to or
      better than a predetermined threshold value, then modify-
      ing the 3-dimensional structural representation and
      predicting the binding free energy between the thus
10   modified substance, B, and the site in the chaperone, and

5)   repeating step 4 until the predicted binding free energy
      determined between the resulting substance, X, and the
      site in the chaperone is equal to or better than the
      predetermined threshold value.

15 It is possible to expand the above-mentioned method with two
further steps, wherein the actual binding free energy is
determined, in order to establish that the experimental
binding free energy also is better than the predetermined
threshold value. By performing the following two steps

20 6)   providing a sample of the chemical substance X and a
      sample of the chaperone and measuring the binding free
      energy between the chemical substance X and the chaperone
      (e.g. by microcalometry as mentioned above), and estab-
      lishing that the measured binding free energy between the
25   chemical substance X and the chaperone is equal to or
      better than the predetermined threshold value, and optio-
      nally

7)   subjecting the substance X to the method mentioned above
      for identifying a substance suitably capable of interac-
30   ting with a chaperone, in order to verify that the sub-
      stance X is a potentially therapeutically useful sub-
      stance capable of interacting with a chaperone,

It is thus verified that the binding free energy between the candidate substance and the chaperone actually is better than the predetermined threshold value. Step 7) further establishes that the candidate substance stands good chances of
5   being therapeutically useful.

The phrase "predicting the binding free energy" is meant to imply that the binding free energy is determined by calculation rather than by performing experimental work determining the actual binding free energy. One (theoretical) way of
10 predicting binding free energy is by performing free energy perturbation (FEP) calculations on the interacting substances, but because of the vast amount of calculations such an approach would have as a result it is preferred that the empirical approximative method described below is employed.

15 The term "better than" is intended to mean that the binding free energy has a value which is higher than the binding free energy which has been chosen as the threshold value, meaning that the ΔG is numerically higher than the threshold value selected. Or in other words: The term is intended to mean
20 that the binding between the substance and the chaperone is more favourable energetically than the situation were the substance and the chaperone are suspended independently in solution.

In order to predict the binding energy in the above-indicated
25 method, according to the invention it is especially preferred to use the following method:

Assessing the average energy difference, $\langle \Delta V_{X-s}^{el} \rangle$, defined as $\langle V_{X-s}^{el} \rangle_B - \langle V_{X-s}^{el} \rangle_A$, between the contribution from polar interactions to the potential energy between the chemical sub-
30 stance X and its surroundings (denoted s) in two states, one state (A) being where the chemical substance is surrounded by solvent, the other state (B) being where the chemical substance, bound to a periplasmic molecular chaperone or an analogue thereof, is surrounded by solvent,

assessing the average energy difference, $<\Delta V^{vdw}_{X-s}>$, defined as $<V^{vdw}_{X-s}>_B$ - $<V^{vdw}_{X-s}>_A$, between the contribution from non-polar interactions to the potential energy between the chemical substance X and its surroundings (denoted s) in two states,

5   one state (A) being where the chemical substance is surrounded by solvent, the other state (B) being where the chemical substance, bound to a periplasmic molecular chaperone or an analogue thereof, is surrounded by solvent, and

calculating the absolute binding free energy as an adjusted
10   combination of the two above-mentioned average energy differences.

In the mathematical equations herein, the symbol < > means molecular dynamics average. The index X-s means compound-solvent (or compound-surrounding), the letter "X" denoting
15   the chemical substance X. Normally the substance X will function as an inhibitor of the binding between the periplasmic chaperone and pilus subunits, but as discussed herein, it is also a possibility that the compound or drug will affect the chaperone in such a way that the binding between pilus
20   subunits and the chaperone is enhanced. The superscript "el" designates the polar or electrostatic energy, while the superscript "vdw" indicates "van der Waals", another designation for the non-polar interactions. The symbol Δ indicates that the quantity in state A is subtracted from the quantity
25   in state B.

In the present context the term "an analogue of a periplasmic molecular chaperone" should be understood, in a broad sense, any substance which mimics (with respect to binding characteristics) an interesting part of a periplasmic molecular
30   chaperone (e.g. the pilus subunit binding part(s)), and the interaction of which with a chemical substance or a group or plurality of chemical substances, e.g. drug candidates, is to be studied. Thus, the analogue may simply be any other chemical compound regarded as capable of interacting with the
35   chemical substance in a manner which mimics the binding

between the chaperone and a pilus subunit in vivo, but most
often the analogue will be a relatively large molecule, in
other words a macromolecule such as a protein or an oligonu-
cleotide, which is relatively large compared to the chemical
5   substance; although the chemical substance interacting with
the analogue, of course, in itself be a macromolecule. In the
present context, the periplasmic molecular chaperone or
analogue thereof is preferably the periplasmic chaperone or
an analogue thereof which exhibits at least one interesting
10 binding characteristic relevant for the assembly of pili.

The basis for the above-indicated approach for determining
the binding free energy is explained in the following:

As a starting point is taken the linear response approxima-
tion for electrostatic forces which for polar solutions as a
15 result yields quadratic free energy functions in response to
the development of charges. This is, e.g., the familiar
result from Marcus' theory of electron transfer reactions
(Marcus, 1964). For a system with two states, A and B, given
by two potential energy functions V_A and V_B one obtains,
20 within the approximation of harmonic free energy functions of
equal curvature, the relationship (see Lee et al., 1992 and
references therein):

$$\lambda - <V_B - V_A>_A - \Delta G_{AB} = <V_A - V_B>_B + \Delta G_{AB} \qquad \text{(a)}$$

where ΔG_{AB} is the free energy difference between B and A, λ
25 the corresponding reorganisation energy and $< >_i$ denotes an
average evaluated near the minimum of the potential i. Thus,

$$\Delta G_{AB} = \tfrac{1}{2}(<\Delta V>_A + <\Delta V>_B) \qquad \text{(b)}$$

where ΔV now denotes the energy difference $V_B - V_A$. If the
hydration of a single ion is considered, this can be shown to
30 give $\Delta G_{sol}^{el} = \tfrac{1}{2}<V_{X-s}^{el}>$, i.e. that the electrostatic contribution
to the solvation energy equals half of the corresponding ion-
solvent interaction energy (Warshel and Russell, 1984; Roux

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et al., 1990). Returning now to the binding problem, this result may be exploited in the following manner: For each solvation process, i.e. solvation of the substance in water and inside the protein, two states are considered where the first has the substance in vacuum and a non-polar cavity (given, e.g., by Lennard-Jones potential) already made in the given environment. The second state corresponds to the intact substance surrounded by water or the solvated protein. The linear response approximation will then again give that $\Delta G^{el}_{bind} \approx \frac{1}{2}<V^{el}_{X\text{-}s}>$, where $V^{el}_{X\text{-}s}$ is the solute-solvent electrostatic term. Hence, the electrostatic contribution to the binding free energy can be approximated by $\Delta G^{el}_{bind} \approx \frac{1}{2}<V^{el}_{X\text{-}s}>$ (where the Δ now refers to the difference between protein and water) and thus obtained from two MD simulations of the solvated substance and of the substance-protein complex.

The validity of the linear response results in the case of ionic solvation has been confirmed, e.g., in the study by Roux et al. (1990). Some additional calculations were also performed on simple systems that corroborate the approximation of equation b. These tests were carried out by comparing the free energy obtained from FEP/MD simulations of charging Na^+ and Ca^{2+} ions in a spherical water system (Åqvist, 1990) with the corresponding $<V^{el}_{X\text{-}s}>$ from 75 ps MD trajectories. This yielded factors relating $<V^{el}_{X\text{-}s}>$ to ΔG^{el}_{sol} of 0.49 for Na^+ and 0.52 for Ca^{2+}, both values being close to the predicted result of $\frac{1}{2}$. A similar test on the charging of a methanol molecule, given by the OPLS potential (Jorgensen, 1986) in water gave a $\Delta G^{el}_{sol}/<V^{el}_{X\text{-}s}>$ ratio of 0.43.

A crucial question is how to account for the contribution of non-polar interactions and hydrophobic effects to the free energy of binding which was termed ΔG^{vdw}_{bind}. In the ideal case, it should be possible to estimate this contribution from the non-polar (or van der Waals) interaction energies. The liquid theories of Chandler and coworkers (Chandler et al., 1983; Pratt and Chandler, 1977) have been successfully used to analyze hydrophobic effects and to calculate free energies of

transfer for some non-polar molecules (Pratt and Chandler,
1977), but no analytical treatment of that kind seems pos-
sible for solvation in an inhomogeneous environment such as a
protein's active site. However, it has been noted that the
experimental free energy of solvation for various hydrocarbon
compounds, such as n-alkanes, depends approximately linearly
on the length of the carbon chain both in their own liquids
as well as in water (Ben-Naim and Marcus, 1984). MD simula-
tions of n-alkanes solvated in water and in a non-polar van
der Waals solvent have been carried out, which indicate that
also the average solute-solvent interaction energies vary
approximately linearly with the number of carbons in the
chain (the relationships being different in different sol-
vents, of course). It thus seem possible that a simple linear
approximation of ΔG_{bind}^{vdw} from $<\Delta V_{X-s}^{vdw}>$ might be able to account
for the non-polar binding contribution. For instance, if σ is
considered some appropriate measure of the size of the solute
and if the solute-solvent van der Waals interaction energies
and the corresponding non-polar free energy contributions
(both in water and protein) depend linearly on σ, such that

$$<V_p^{vdw}> = \alpha_p\sigma, \quad <V_w^{vdw}> = \alpha_w\sigma, \quad \Delta G_p^{vdw} = \beta_p\sigma \text{ and } \Delta G_w^{vdw} = \beta_w\sigma$$

then $\Delta G_{bind}^{vdw} = \frac{\beta_p - \beta_w}{\alpha_p - \alpha_w}<\Delta V_{X-s}^{vdw}>$ is obtained. Since it seems diffi-
cult to derive a factor relating the two quantities in a
reliable way from purely theoretical considerations, the
approach is taken to empirically try to determine such a
relationship which is capable of reproducing experimental
binding data. Thus, the free energy of binding is in one
embodiment of the invention approximated by

$$\Delta G_{bind} = \frac{1}{2}<\Delta V_{X-s}^{el}> + \alpha<\Delta V_{X-s}^{vdw}> \tag{1}$$

the parameter α being determined by empirical calibration.

Although, as discussed above, a theoretical prediction of the
coefficient for $<\Delta V_{X-s}^{el}>$ is $\frac{1}{2}$, it may be practically useful to

also treat this coefficient as an empirical parameter. This would lead to the free energy of binding being approximated by

$$\Delta G_{bind} = \beta <\Delta V^{el}_{X\text{-}s}> + \alpha <\Delta V^{vdw}_{X\text{-}s}> \qquad (1b)$$

where both parameters, α and β, are determined by empirical calibration.

Finally, in some cases, it seems suitable to add an additional constant term to Equation 1, so that the equation becomes

$$\Delta G_{bind} = \beta <\Delta V^{el}_{X\text{-}s}> + \alpha <\Delta V^{vdw}_{X\text{-}s}> + c \qquad (2)$$

where c is a constant reflecting extrapolation to zero size of the chemical substance, that is, where the regression line is distinctly offset from origin when moving towards zero size of the chemical substance. The parameter c may also be used to correct for possible systematic errors due to e.g. the neglect of induced polarisation, possible force field deficiencies etc. In these cases, c will normally assume a value between -10 and 10 kcal/mol, typically between -3 and 3 kcal/mol, such as between -2 and 2 kcal/mol, e.g. between -1 and 1 kcal/mol. However, it is anticipated that in many cases, c can suitably be set to zero, as the extent of deviation will be of minor importance for the usefulness of the predicted values.

If also the electrostatic coefficient i treated as an empirical parameter, the approximation of the binding free energy assumes its most general form, namely

$$\Delta G_{bind} = \beta <\Delta V^{el}_{X\text{-}s}> + \alpha <\Delta V^{vdw}_{X\text{-}s}> + c \qquad (2b)$$

where now both α, β and c are to be determined by empirical calibration.

While the solvent used in the above method is suitably and
most often an aqueous solvent like water, it is within the
scope of the invention to take any other suitable solvent as
a starting point, including, e.g., methanol, ethanol, aceto-
5   ne, acetonitrile, chloroform, hexane, etc., or mixtures
thereof or combinations of such solvents or mixtures thereof
with water. The selection of the solvent will be of little
importance to the predicted values as long as the solvent is
one which is able to dissolve or solvate the receptor molecu-
10   le and the substance (in the present context this means that
a sufficient amount of the periplasmic molecular chaperone or
analogue thereof can be homogeneously mixed with the solvent
without precipitation so as to allow the determination of
binding energies by some suitable method), but there may be
15   cases where it is advantageous to modify the solvent environ-
ment (e.g. by modulating the ionic strength) in which the
interaction of the substance and the receptor molecule is to
take place. If the environment in which the interaction
between the chemical substance, such as a drug, and a peri-
20   plasmic molecular chaperone or an analogue thereof is to take
place in the actual use of the drug is the human body, it
might be particularly suitable to imitate e.g. human plasma
as the solvent.

A thorough discussion of the above-referenced method for
25   determining the binding free energy between two molecules can
be found in International Patent Application No.
PCT/IB94/00257 and in Åqvist et al, 1994. These two documents
are hereby incorporated by reference.

The above referenced method for determining the binding free
30   energy has been employed in example 3 in order to identify
compounds which stands a high chance of binding to the bin-
ding site of PapD; this means that calculations as the above
described have been performed as the last theoretical step
before compounds have actually been synthesized.

It will be understood that the above mentioned methods for identifying substances capable of interacting with chaperones will prove especially efficient in identifying substances which are of potential pharmaceutical value if the site to
5   which they bind is known to be involved in pilus assembly.

Therefore, it is preferred that the site with which the substance may potentially interact, and to which the binding free energy is predicted, is the pilus subunit binding part of a molecular chaperone, such as the pilus subunit binding
10   site of a molecular chaperone selected from the group consisting of PapD, FimC, SfaE, FaeE, FanE, Cs3-27, F17D, ClpE, EcpD, Mrkb, FimB, SefB, HifB, MyfB, PsaB, PefD, YehC, MrpD, CssC, NfaE, AggD and Caf1M, or an analogue of such a pilus subunit binding site, since the pilus subunit binding sites
15   in these chaperones show extensive homologies. It is especially preferred that the binding site is the pilus subunit binding site of PapD or an analogue thereof.

As will appear from the examples, an important part of the chaperone binding motif has been discovered and a peptide
20   corresponding to this motif has been synthesized and co-crystallized with PapD to provide a structural basis for the mechanism of action of PapD. The molecular details of the PapD-adhesin recognition interface clearly demonstrate the function of the conserved cleft in the entire pilus chaperone
25   superfamily in subunit binding and in shuttling virulence determinants to the surface of pathogenic bacteria. The PapD-peptide crystal structure essentially represents a "snapshot" of a fundamental process in bacterial pathogenesis: the interaction of an adhesin with a chaperone, which is a pre-
30   requisite to adhesin presentation on the microbial surface.

Thus, the inventors of the present invention have by the use of X-ray crystallography elucidated the mechanism of binding between PapD and the pilus subunit PapG thereby identifying an essential part of a defined binding site responsible for
35   the binding between pilus subunits and their periplasmatic

chaperones, and thus providing a method to enable drug design
of chaperone inhibiting anti-bacterial compounds.

Having determined the location of a promising binding site
for inhibitory ligands as described above (see details in
5   examples 1 and 2), the computer programs "PLIM" and
"PLIM_DBS" (developed by Symbicom AB) have been used to find
templates for families of compounds capable of binding to the
binding site.

PLIM is a Protein Ligand Interaction Modeller that constructs
10 putative ligands for a protein using thermodynamic criteria.
It calculates the energy of interaction between the protein
and sample probes that are successively placed at different
points on a regular grid around the molecule. For each posi-
tion and orientation the interaction energy between the probe
15 and the atoms of the protein is calculated. The energies are
stored, and the best positions for a particular probe are
written out (the basic calculations are described by Goodford
(1985) and Boobbyer (1989) and implemented in the commercial-
ly available program GRID; the PLIM implementation is some-
20 what different in that the energy values are converted to
discrete points that are associated with the chemical probe,
enabling easy output to e.g. data base searching programs).
The program then builds up the ligand by incorporating
selected probe atoms at positions of energy minima on the
25 grid. The user selects which atoms and groups should be used
as probes, and which criteria should be used to determine
those that will be incorporated into the ligand. The energy
is calculated as the sum of electrostatic, Van der Waals and
hydrogen-bonding contributions as described herein.

30 The PLIM runs result in a number of suggested positions and
orientations of favourable chemical groups in the region near
the binding site. These groups which have physical properties
like charge, hydrogen bonding directionality and extended
atom radia, will hereafter be denoted "site points".

A search for potential ligands is then made by searching a
database for known molecular structures that match the posi-
tions of these groups of site points, using PLIM_DBS.

The core of PLIM_DBS is an algorithm for subgraph isomorphism
5   (cf. Ullman (1976) and Brint (1987)), where three sitepoints
are represented as a distance matrix ("the pattern matrix").
The program looks for this distance pattern in the distance
matrix formed from every entry in the database. If the pat-
tern is found, the entry is superimposed on to the sitepoints
10   and if the corresponding atom types match the entry and its
orientation is saved in a hit-list. Added to this basic
scheme a number of options regarding surface complementarity
can be used, i.e. only entries which are matching the protein
surface with respect to hydrophobic and steric properties are
15   saved.

PLIM_DBS is thus a database searcher which hunts through a
collection of 3-dimensional molecule coordinate sets, looking
for entries that contain a certain pattern of atoms. This
pattern is specified in terms of atom type, and of spatial
20   position and orientation; for instance a search may be made
for compounds containing an sp3 carbon atom that is 4.2 Å
from a sp2 oxygen and 5.1 Å from a hydroxyl group that in
turn is 5.6 Å from the oxygen. The strictness of the search
can be adjusted by the user by varying the tolerance on the
25   distance criteria and the atom-type matching, determining,
for instance, whether a sp2 carbon that is a little more than
4.2 Å from an oxygen should be considered as a hit. Those
hits that are found are then ranked according to a score that
reflects how well the target atoms superimpose on the real
30   molecule, and also on how complementary the molecular surface
of the compound is to that of the binding pocket of the
protein.

The result from a PLIM_DBS search is a list of molecular
structures and their atomic coordinates, superpositioned on
35   to the sitepoints, and given a score ("goodness of fit").

The procedure does not try to optimize the positioning of the structures, nor does it perform any molecular mechanics or dynamics calculations. Both protein and the extracted structures are treated as rigid bodies.

5 The structures from the database search are displayed in the context of the protein and its surface on a graphics system using a commonly available molecular modelling package. Usually the structures show some unfavourable interactions with the protein, or lack groups to fill out e.g. hydrophobic
10 pockets. Hence, the structures form the database search are regarded as templates, to be modified and improved by an organic chemist. This process also involves choosing compounds which are easy to synthesize, which is of particular interest if the synthesis capacity is limited.

15 The best of these database hits are thus examined visually using a computer-graphic modelling system, and the most promising of these are selected according to a wealth of physico-chemical reasoning.

The templates are modified using a small molecule 3D builder
20 (MacMimic). Each template gives rise to a compound class, e.g. denoted "hdo". Each modification assigned a specific number (e.g. hdo_3) and the coordinates and a description are stored in a tree structure, using the program ARVS_JAKT developed by Symbicom. The design is performed in a collabo-
25 ration between protein structure experts and organic chemists, in order to provide the best tools possible for the chemists who will actually synthesize the compounds.

The efficacy of these modifications is finally assessed using molecular-dynamics free energy calculations as described
30 herein to study the stability of the protein-ligand complex (Åqvist et al., 1994; Åqvist and Medina, 1993).

In order to maximize the efficiency of the above-mentioned methods for identifying/designing substances which are

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capable of interacting with a molecular chaperone, it is preferred that the substance A is likely to be a substance which is capable of binding to the selected binding site.

In view of the above-described *modus operandi* for selecting
5 substances which should interact with chaperones like *PapD*, this can, according to the invention, be accomplished when the substance A is selected by performing the following steps:

- co-crystallizing the periplasmic molecular chaperone or
10 the analogue thereof with a ligand capable of interacting with a site in the periplasmic molecular chaperone or the analogue thereof and establishing the three-dimensional conformation of the periplasmic molecular chaperone or the analogue thereof and the ligand when interacting by
15 means of X-ray crystallography,

- using the above-established conformation of the periplasmic molecular chaperone or the analogue thereof to establish a 3-dimensional representation of the site in the periplasmic molecular chaperone or the analogue thereof
20 interacting with the ligand during binding,

- selecting a number of distinct chemical groups, X1, and determining the possible spatial distributions of the X1 chemical groups which maximizes the binding free energy between the chemical groups and the site in the chaperone
25 or the analogue interacting with the ligand,

- extracting, from a database comprising three-dimensional representations of molecules, a molecule which has the X1 chemical groups in the possible spatial distributions determined above,

30 - optionally modifying the 3-dimensional representation of the molecule extracted from the database, and

identifying the optionally modified molecule as the substance A.

According to the invention the above-indicated steps are especially preferred when the ligand is a pilus subunit or a
5   part thereof with which the chaperone normally interacts during transport of the pilus subunit through the periplasmic space and/or during pilus assembly.

By the use of the above-mentioned method for identifying substances capable of interacting with the periplasmic chape-
10 rones, several classes of substances have been identified which have proved promising in the design phase.

Drug design efforts for inhibitors/enhancers to PapD have concentrated on the region of the molecule where the G-peptide is observed to bind. This region will now be
15 described in detail, using one of the putative inhibitors (bpy_9, see below) as a reference structure.

The binding site is dominated by the central charged side chains of Arg-8 and Lys-112, which bind to the sulphate moiety of bpy_9. Adjacent to this is a small, shallow
20 hydrophobic pocket formed by the side-chains of Ile-154, Ile-194, and Thr-7, against which the 2-ethyl group of bpy_9 packs (Pocket 1). A group as large as a phenyl ring could be accommodated here, attached to sugar position 2, and with possible substituents that could receive or donate a hydrogen
25 bond to Thr-7, or donate to the backbone carbonyl of 198.

There is also a larger pocket comprising residues Leu-4, Ile-111, Thr-7 and Thr-109, in which the phenyl ring of bpy_9 nestles (Pocket 2). A group as large as naphthalene could be substituted on the 6-position of the carbohydrate scaffold to
30 fill this sub-site, with substituents that could form a hydrogen bond to Thr-109, or to any of the polar backbone atoms of residues Leu-4, Arg-6, or Lys-110. Then there is a long, shallow patch that includes Tyr-87, and the aliphatic

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regions of Lys-110 and Lys-112, which could accommodate a
tricyclic system such as 2-phenanthryl substituted on posi-
tion 3 of the sugar (Patch 3). Substituents to hydrogen bond
to Tyr-87 or the backbone of Lys-110 can be considered, as
5   well as negatively charged groups to complement the side
chains of Lys-110 and Lys-112. Tyr-87 is a potential charge-
transfer donor to a electron deficient π-system such as a
nitroaryl.

Models of several homologous chaperones (*SfaE*, *MrkB*, *HifB* and
10   *FimC*) have been made from the *PapD* structure, and differences
between the model structures has influenced the design of
inhibitors, such that proposed ligands should bind to all the
structures looked at. For example, it is tempting to comple-
ment the charged side chain of Arg-200 with an acidic group
15   in the ligand, but since two of the other structures have an
Asp at this position, the residue is not considered as a good
candidate. Arg-8 is fully conserved, as is Lys-112, Thr-7 and
Ile-11. Tyr-87 becomes a Trp in three structures, but the
overall nature of patch 3 is not changed by this. *PapD* is
20   actually alone with its Thr-Lys sequence at 109-110, all of
the four other structures being Ser-Arg here, but again,
these conservative changes do not significantly alter design
criteria.

Although substances which interact with the binding site
25   responsible for binding to G-proteins are obvious candidates
as inhibitors/enhancers of periplasmic chaperones, it will be
understood that molecules capable of interacting with other
sites in periplasmic chaperones are interesting in this
aspect, too. It is highly possible that an interaction with
30   another site than the one binding G-protein in *i.e.* *PapD* may
cause *PapD* to either be prevented, inhibited or enhanced in
its action as a periplasmic chaperone.

As mentioned above, a family of substances (called the bpy
family herein) is an important aspect of the invention. Thus,

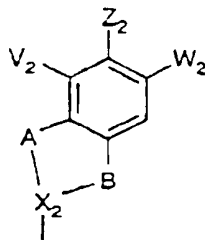the invention relates to novel compounds of the general formula:

I
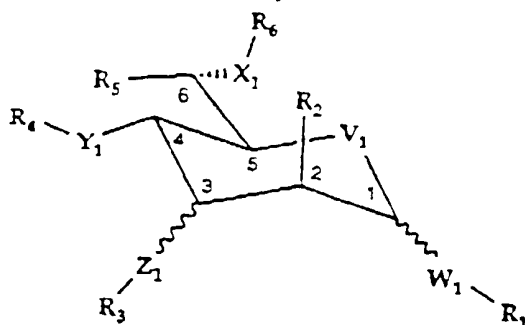
wherein

V_1 is O, S, SO, SO_2, CH_2, C(OH)H, CO or CS;

5   W_1 is O, S, SO_2, SO_3, CH_2 or NH;

R_1 is H; C_{1-24} alkyl, C_{1-24} alkenyl or C_{1-24} alkynyl, which
alkyl, alkenyl and alkynyl may be substituted with one or
more substituents independently selected from OH, $-CONH_2$,
$-CSNH_2$, $-CONHOH$, $-CSNHOH$, $-NHCHO$, $-NHCONH_2$, $-NHCSNH_2$,
10   $-NHSO_2NH_2$ and $-SO_2NH_2$; acyl; or $-(CH_2CH_2O)_s$-H, wherein s =
1,2,3;

R_2 is a group of the formula

wherein
        A is $-CH-(CH_2)_n-$, or $-CH=CH-(CH_2)_{n-1}-$ (n>0) or
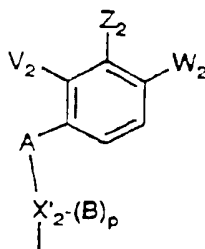
$$-CH-(CH_2)_n-;$$
$$\|$$
$$Y_2$$

B is $-(CH_2)_m-$ or $=CH-(CH_2)_{m-1}-$ $(m>0)$;

X_2 is N, CH or C (when B is $=CH-(CH_2)_{m-1}-$; and

Y_2 is O, S, NH, H_2 or H $(n=1)$; and

$4>m+n>0$, $n<3$, and $m<3$;

or R_2 is a group of the formula

wherein

A is $-CH-(CH_2)_n-$, or $-CH=CH-(CH_2)_{n-1}-$ $(n>0)$ or
$-CH-(CH_2)_n-$;
$$\|$$
$$Y_2$$

B is $-(CH_2)_m-$ or $=CH-(CH_2)_{m-1}-$ $(m>0)$; and

X'_2 is O, NH, CH_2 or S (when $p=0$); N or CH $(p=1)$;
or C (when $p=1$ and B is $=CH-(CH_2)_{m-1}-$);

V_2, Z_2 and W_2 are independently H, OH, $-CONH_2$,
$-CSNH_2$, $-CONHOH$, $-CSNHOH$, $-NHCHO$, $-NHCONH_2$,
$-NHCSNH_2$, $-NHSO_2NH_2$, $-SO_2NH_2$, or V_2 and Z_2, or Z_2
and W_2 together form $-NHC(O)NH-$, $-C(O)NHC(O)-$,
$-NHS(O_2)NH-$, $-C(O)NHO-$, $-C(S)NHO-$, $-S(O_2)NHO-$,
or $-S(O_2)NHC(O)-$;

$4>m+n>0$, $n<3$, and $m<3$;

or R_2 is a group $-W_5-(C_{1-5}$ alkyl or C_{2-5} alkenyl or C_{2-5} alkynyl) wherein W_5 is a bond or is selected from $-O-$, $-S-$, $-SO_2-$,

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and -NHC(O)-, and the C_{1-5} alkyl, C_{2-5} alkenyl or C_{2-5} alkynyl
moiety may be substituted with up to three groups selected
independently from OH, -$CONH_2$, -$CSNH_2$, -CONHOH, -CSNHOH,
-NHCHO, -$NHCONH_2$, -$NHCSNH_2$, -$NHSO_2NH_2$ and -SO_2NH_2;

5   -Z_1-R_3 is -SO_2(OH), -PO(OH)$_2$, -OSO_2(OH), -$NHSO_2$(OH),
-NH-CO-COOH, -SPO(OH)$_2$, -CH_2COOH, tetrazol-5-yl or tetrazol-
5-ylmethyl, or salts thereof;

or Z_1 is -O-, -S-, -NH-, or -CH_2-, and R_3 is a group of the
formula:

10   D is -CH_2-, -CO-, -SO_2-, -NH-SO_2-, -NH-CO-, -O-PO(OH)- or
a salt thereof;

Z_3 is H, OH, -$CONH_2$, -$CSNH_2$, -CONHOH, -CSNHOH, -NHCHO,
-$NHCONH_2$, -$NHCSNH_2$, -$NHSO_2NH_2$, -SO_2NH_2, -SO_2(OH),
-PO(OH)$_2$, -OSO_2(OH), -$NHSO_2$(OH), -COOH, tetrazolyl-5-yl
15   or tetrazolyl-5-ylmethyl or a salt thereof,

with the proviso that when D is $-CH_2-$, $-CO-$, $-SO_2-$,
$-NHSO_2-$ or $-NHCO-$, then Z_3 is $-SO_2(OH)$, $-PO(OH)_2$,
$-OSO_2(OH)$, $-NHSO_2(OH)$, $-COOH$, tetrazolyl-5-yl or tetrazolyl-5-ylmethyl or a salt thereof;

5   X_3 and Y_3 independently are H, NO_2, SO_2NH_2, $CONH_2$, CF_3 or
F; and

U_4-W_4 is $-CHCH-$, $-CH_2CH_2-$, $-C(OH)CH-$, $-CHC(OH)-$,
$-CH(OH)CH_2-$, $-CH_2CH(OH)-$, $-CH(OH)CH(OH)-$, $-C(O)NH-$,
$-NHC(O)-$;

10   Y_1 is $-O-$ or $-S-$;

R_4 is H or, when Y_1 is S, $S(CH_2)_qN(R_9)_3^+$ and q is an integer
2-4, where R_9 is H or CH_3;

R_5 is H; C_{1-6} alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl, and the C_{1-6}
alkyl, C_{2-6} alkenyl or C_{2-6} alkynyl moiety may be substituted
15 with OH, $-CONH_2$, $-CSNH_2$, $-CONHOH$, $-CSNHOH$, $-NHCHO$, $-NHCONH_2$,
$-NHCSNH_2$, $-NHSO_2NH_2$ or $-SO_2NH_2$; or aryl, aryl(C_{1-2})alkyl,
heterocyclyl, or heterocyclyl(C_{1-2})alkyl which may optionally
be substituted in the aryl or heterocyclyl moieties with one,
two or three substituents selected independently from OH, F,
20 Cl, NH_2, $CONH_2$, NHCOH, and SO_2NH_2;

X_1 is $-O-$, $-S-$ or $-NH-$;

R_6 is H or, when X_1 is NH, acyl, HOCNH-Val-Met-, HOCNH-Ile-
(S,S)-dioxo-methionyl- or HOCNH-Val-(pyran-4-on-2-yl)-ala-
nyl-;

25   or a salt of such a compound.

Another family of substances called the hdo-family has also
been synthesized. Hence, the invention also relates to novel
compounds of the general formula

$$R_{14}Z_{12} \qquad \overset{Z_{13}R_{15}}{\underset{R_{13}}{\diagup}} \qquad X$$
$$R_{12} \qquad Z_{11}R_{11}$$

II

wherein

X is O, P, P(O), S, SO, SO_2, CH_2, C(OH)H, or a group NQ_{11}, wherein Q_{11} is H, OH, C_{1-24} acyl or C_{1-24} alkyl;

5   Z_{11} is a bond, O, CH_2, S, SO, SO_2, or a group NQ_{12}, wherein Q_{12} is H, C_{1-24} acyl or C_{1-24} alkyl;

R_{11} is H; C_{1-24} alkyl, C_{2-24} alkenyl, or C_{2-24} alkynyl, which may be substituted with one or more substituents independent-
ly selected from -OH, -COOH, -F, -Cl, -CONH$_2$, -CSNH$_2$,
10   -CONHOH, -CSNHOH, -NHCHO, -NHCONH$_2$, -NHCSNH$_2$, -NHSO$_3$NH2 and -SO$_2$NH$_2$; acyl; or -(CH$_2$CH$_2$O)$_s$-H, wherein s = 1, 2, or 3;

or R_{11} is CH=CH-(CH$_2$)$_{n'}$-Q$_{13}$ or -(CH$_2$)$_{n'}$-Q$_{13}$, wherein Q_{13} is an aryl or a heteroaryl group substituted with -OH, -COOH, -F,
-Cl, -CONH$_2$, -CSNH$_2$, -CONHOH, -CSNHOH, -NHCHO, -NHCONH$_2$,
15   -NHCSNH$_2$, -NHSO$_3$NH2 and -SO$_2$NH$_2$, and wherein n' ≥ 0;

R_{12} and R_{13} are independently OH, H, F, Cl, OW$_{11}$, or O(CO)W$_{11}$, wherein W_{11} is C_{1-24} alkyl, C_{2-24} alkenyl or C_{2-24} alkynyl, or an aryl or a heteroaryl group substituted with -OH, -COOH,
-F, -Cl, -CONH$_2$, -CSNH$_2$, -CONHOH, -CSNHOH, -NHCHO, -NHCONH$_2$,
20   -NHCSNH$_2$, -NHSO$_3$NH2 and -SO$_2$NH$_2$;

Z_{12} is a bond, O, S, or CH$_2$;

R_{14} is -(CH$_2$)$_{n''}$-Q$_{14}$, wherein Q_{14} is an aryl group or a hete-
roaryl group substituted with -OH, -COOH, -F, -Cl, -CONH$_2$,
-CSNH$_2$, -CONHOH, -CSNHOH, -NHCHO, -NHCONH$_2$, -NHCSNH$_2$,
25   -NHSO$_3$NH2 and -SO$_2$NH$_2$, and wherein n'' = 0, 1, 2, or 3;

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Z_{13} is a bond, O, CH_2, S, SO, SO_2, $NQ_{14}Q_{15}$, wherein Q_{14} is H, C_{1-24} acyl or C_{1-24} alkyl, and Q_{15} is CO or -C(O)W_{12}, wherein W_{12} is O or NW_{13}, wherein W_{13} is H, OH, C_{1-24} acyl or C_{1-24} alkyl;

5   R_{15} is H; C_{1-24} alkyl, C_{2-24} alkenyl or C_{2-24} alkynyl, which alkyl, alkenyl and alkynyl may be substituted with one or more substituents independently selected from, -OH, -COOH, -F, -Cl, -$CONH_2$, -$CSNH_2$, -CONHOH, -CSNHOH, -NHCHO, -$NHCONH_2$, -$NHCSNH_2$, -$NHSO_3NH2$ and -SO_2NH_2; acyl or -$(CH_2CH_2O)_s$-H, where-
10 in s = 1, 2, or 3;

or R_{15} is CH=CH-$(CH_2)_{n'}$-Q_{13}, or -$(CH_2)_{n'}$-Q_{13}, wherein Q_3 is as defined above and wherein n' $\geq$ 0;

or a salt of such a compound.

In the present context, the terms "C_{1-5}, C_{1-6} and C_{1-24} alkyl"
15 is intended to mean alkyl groups with 1-5, 1-6 and 1-24 carbon atoms, respectively, which may be straight or branched or cyclic such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert.butyl, hexyl, octyl, dodecyl, cyclopentyl, cyclohexyl, etc.

20 Further, as used herein, the terms "C_{2-5}, C_{2-6} and C_{2-24} alke-nyl" is intended to mean mono- or polyunsaturated alkyl groups with 2-5 and 2-24 carbon atoms, respectively, which may be straight or branched or cyclic in which the double bond(s) may be present anywhere in the chain or the ring, for
25 example vinyl, 1-propenyl, 2-propenyl, hexenyl, decenyl, 1,3-heptadienyl, cyclohexenyl etc. Some of the substituents exist both in a cis and a trans configuration. The scope of this inventions comprises both the cis and trans forms.

In the present context, the terms "C_{2-5}, C_{2-6} and C_{2-24} alky-
30 nyl" is intended to mean a straight or branched alkyl group with 2-5 and 2-24 carbon atoms, respectively, and incorporat-

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ing one or more triple bond(s), e.g. ethynyl, 1-propynyl, 2-propynyl, 2-butynyl, 1,6-heptadiynyl, etc.

The terms "C_{1-6} and C_{1-24} alkoxy" designate alkyl groups as defined above comprising an oxy function.

5   In the present context, the term "aryl" is intended to mean phenyl and naphthyl. The term "heteroaryl" is intended to mean a cyclic aromatic system, wherein at least one non-carbon atom contributes to the π bonding system.

Examples of substituted aryl groups are: 3-nitrophenyl, 3-
10 hydroxyphenyl, 4-hydroxyphenyl, 3,4-dihydroxyphenyl, 3-car-
boxamidophenyl, 3-formamidylphenyl, 3-acetamidylphenyl, 3-
fluoronapht-2-yl, 7-fluoronaphthyl, 3,7-difluoronaphthyl, 3-
hydroxynaphthyl, 7-hydronaphthyl, 3,7-dihydroxynaphthyl, 3-
fluoro-7-hydroxynaphthyl, 7-fluoro-3-hydroxynaphthyl, 4-
15 fluoronaphth-2-yl, 6-fluoronaphth-yl, 8-fluoronaphth-2-yl,
4,6-difluoronaphth-2-yl etc.

Examples of heterocyclic and heteroaryl groups are pyrrolyl, furanyl, 2,3-dihydrofuranyl, tetrahydrofuranyl, thienyl, 2,3-dihydrothienyl, tetrahydrothienyl, imidazolyl, oxazolyl,
20 thiazolyl, pyrazolyl, indolyl, pyrazinyl, dioxolanyl, dioxan-yl, 1,3,5-trioxanyl, tetrahydrothiapyranyl, dithiolanyl, pyrazolidinyl, iminazolidinyl, *sym*-triazinyl, *sym*-tetrazinyl, quinazolinyl, pteridinyl, isoindolyl, 1,2,4-triazolyl, 1,2,3-triazolyl, benzimidazolyl, indazolyl, benzofuranyl, isoben-
25 zofuranyl, benzothiophenyl, thienothiophenyl, isoxazolyl, 1,2,5-oxadiazolyl, isothiazolyl, 1,3,4-thiadiazolyl, benzoxa-zolyl, benzothiazolyl, azaindolyl, oxoindolyl, hydroxyindo-lyl, N-oxyisoquinolyl etc.

In the present context, the term "acyl" (e.g. C_{1-24} acyl) is
30 intended to designate the acyl residue of a carboxylic acid or a sulphonic acid moiety comprising a carbonyl or sulphonyl group and an organic moiety. Examples of acyl groups include C_{1-24} alkanoyl (e.g. formyl, acetyl, propionyl, butyryl,

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isobutyryl, valeryl, isovaleryl, pivaloyl and hexanoyl),
C_{1-24} alkenoyl (e.g. acryloyl, metacryloyl, crotonoyl, iso-
crotonoyl, 2-pentenoyl, 3-pentenoyl, 2-methylpentenoyl, 3-
pentenoyl, 3-phenylpropenoyl, 2-phenyl-trans-propenoyl, 2,4-
5   hexadienoyl), C_{1-24} alkynoyl (e.g. propyonyl, 2-butynoyl, 3-
butynoyl, 2-methyl-3-butynoyl, 2,2-dimethylbutynoyl, 2-penty-
noyl, 3-pentynoyl, 2-pentyn-4-trans-enoyl), C_{1-24} alkoxycar-
bonyl (e.g. methoxycarbonyl, ethoxycarbonyl, propoxycarbonyl,
butoxycarbonyl and t-butoxycarbonyl), C_{1-24} alkenyloxycarbon-
10   yl (e.g. cis-2-butenyloxycarbonyl, 1-methyl-2-propenyloxycar-
bonyl, 1,1-dimethyl-2-propenyloxycarbonyl, trans-2-butenyl-
oxycarbonyl), aroyl (e.g. benzoyl), heterocyclylcarbonyl
(e.g. 2-furoyl, 3-furoyl, 2-furanoyl, 3-furanoyl, 2-pyrrol-
carboxyl, 3-pyrrolcarboxyl, 2-thenoyl, 3-thenoyl, 2-indolcar-
15   boxyl, 3-indolcarboxyl, 1-naphthanoyl and 2-naphthanoyl),
etc.

The term "salt" is intended to comprise a salt such as an
organic acid addition salt (e.g. acetate, valerate,
salicylate, galacturonate, gluconate, tannate, triflouroace-
20   tate, maleate, tartrate, methanesulfonate, benzenesulfonate,
formiate, thiocyanate and toluenesulfonate), an inorganic
acid addition salt (e.g. hydrochloride, hydrobromide, hydro-
iodide, dihydrochloride, dihydrobromide, dihydroiodide,
sulphate, hydrogensulphate, halosulphate such as iodosul-
25   phate, nitrate, phosphate, and carbonate) or a salt with an
amino acid (e.g. arginine, aspartic acid and glutamic acid)
or a metal salt such as an alkali metal salt (e.g. sodium
salt and potassium salt) and an earth alkali metal salt (e.g
calcium salt and magnesium salt), an ammonium salt, an
30   organic alkali salt (e.g. trimethylamine salt, triethylamine
salt, pyridine salt, picoline salt, dicyclohexylamine salt
and N,N'-dibenzylethylenediamine salt), and hydrates thereof.

When the substituent R_5 designates heterocyclyl, it is pre-
ferred that the substituent designates a heterocyclyl group
35   of the formula
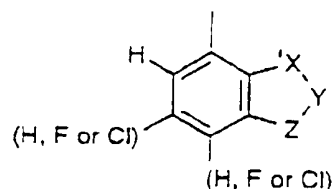
wherein 'X is -CH-, -CH$_2$-, -O-, -N-, -S-, -S→O, -N→O or -CO-,
Y is -CH- or -NH-, and Z is -CH-, -CH$_2$-, -O-, -S-, -N-, -CO-,
-S→O or -N→O, especially a group selected from the group
consisting of inden-7-yl, benzofuran-4-yl, isobenzofuran-4-
5   yl, thionaphthen-4-yl, isothionaphthen-4-yl, 2-oxo-inden-7-
yl, 2-oxo-inden-4-yl, inden-4-yl, benzofuran-7-yl, isoben-
zofuran-7-yl, thionaphten-7-yl, isothionaphthen-7-yl, 1-oxo-
thionaphthen-4-yl, 1-oxo-thionaphthen-7-yl, anthran-4-yl,
anthran-7-yl, thioanthran-4-yl, thioanthran-7-yl, benzthio-
10 zol-4-yl, benzthiozol-7-yl, 2H-2-isobenzo-1,3-dion-7-yl,
isobenzofuran-5-yl, isobenzofuran-6-yl, 3H-2-oxo-benzofuran-
5-yl, 3H-2-oxo-benzofuran-6-yl, 3H-2-oxothionaphthen-5-yl,
3H-2-oxothionaphthen-6-yl, indol-5-yl, indol-6-yl, 3H-2-
oxoindol-5-yl, 3H-2-oxoindol-6-yl, 3H-2-oxobenzoxazol-5-yl,
15 3H-2-oxobenzoxazol-6-yl, benzothiazol-5-yl, benzothiazol-6-
yl, 2-oxobenzo-1,3-dithiol-5-yl, 2-oxobenzo-1,3-dithiol-6-yl,
3H-2-oxobenzimidazol-5-yl, 3H-2-oxobenzimidazol-6-yl, ben-
zoxathiol-5-yl, benzoxathiol-6-yl, 3H-2-oxobenzthiazol-5-yl
and 3H-2-oxobenzthiozol-6-yl.

20 It is also preferred that the substituent R$_5$ is a group of
the formula
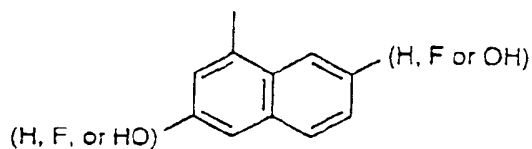
or that R_5 is a group of the formula
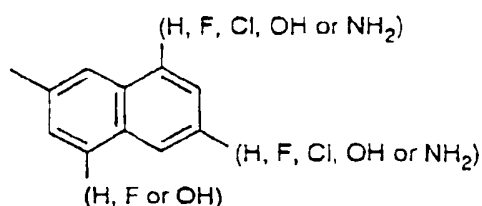
With respect to the substituent R_2 being heterocyclyl, it is especially preferred that it is selected from the group consisting of isobenzofuran-5-yl, isobenzofuran-6-yl, 3H-2-oxo-benzofuran-5-yl, 3H-2-oxo-benzofuran-6-yl, 3H-2-oxothionaphthen-5-yl, 3H-2-oxothionaphthen-6-yl, indol-5-yl, indol-6-yl, 3H-2-oxoindol-5-yl, 3H-2-oxoindol-6-yl, 3H-2-oxobenzoxazol-5-yl, 3H-2-oxobenzoxazol-6-yl, benzothiazol-5-yl, benzothiazol-6-yl, 2-oxobenzo-1,3-dithiol-5-yl, 2-oxobenzo-1,3-dithiol-6-yl, 3H-2-oxobenzimidazol-5-yl, 3H-2-oxobenzimidazol-6-yl, benzoxathiol-5-yl, benzoxathiol-6-yl, 3H-2-oxobenzthiazol-5-yl and 3H-2-oxobenzthiozol-6-yl.

The exact number of substituents present on an alkyl, alkenyl or alkynyl moiety R_1 will be dependent on the length of the carbon chain, the purpose of the substituents being to cause the entire group R_1 to be compatible with water since, in the chaperone-ligand-complex such as the *PapD*-ligand complex, the group R_1 will extend into the surrounding aqueous medium. Thus, for a fairly short carbon chain such as up to four carbon atoms, it is contemplated that one of the above polar

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substituents will be sufficient, in particular when the
substituent is located terminally whereas, for longer chains,
a larger number of substituents, such as a substituent for
every other carbon atom, may be required.

5   The 4,6-O-(4'-Methoxy)phenylmethylidene-α-D-glucohexopyrano-
side or 4,6-O-(4'-Methoxy)phenylmethylidene-β-D-glucohexopy-
ranoside glycosides:

A
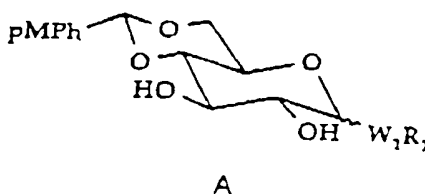
(used here as preferred examples, but other arylmethylidene
or vinylidene acetals may be used) can be prepared as fol-
10 lows: Peracylated glucose is reacted with, e.g. hydrogen
bromide or hydrogen chloride in a suitable solvent such as,
e.g. acetic acid or dichloromethane, to form per-O-acylated
glycosyl bromide or chloride (O-acylation and glycosyl halide
synthesis: M. L. Wolfrom and A. Thompson, *Methods in Carbohy-*
15 *drate Chemistry*, Vol. 2, 211-215, ed. by R.L. Whistler and L.
Wolfrom, Academic Press, New York, 1963; G.Hewit and G.
Fletcher Jr., *ibid*, 226-228; and R.U. Lemieux, *ibid*, 223-224)

The suitably protected, when necessary, aglycone alcohol or
thiol (H-W₁R₁-PG₁ or H-W₁R₁) (protecting groups: *Protective*
20 *Groups in Organic Synthesis*, Editors T. W. Greene and P. G.
M. Wuts, John Wiley & Sons, Inc., New York, 1991) is reacted
with the per-O-acylated glucose using a Lewis acid such as
borontrifluoride etherate (R. J. Ferrier and R. H. Furneaux,
Carbohydr. Res., 52 (1976) 63-68; J. Dahmén, T. Frejd, G.
25 Grönberg, T. Lave, G. Magnusson, and G. Noori, *Carbohydr. Res*
116 (1983) 303-307) or trimethylsilyltrifluoromethanesulpho-
nate (T. Ogawa, K. Beppu, S. Nakabayashi, *Carbohydr. Res.*, 93
(1981) C6-C9) as promotors. The reaction is carried out in a

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suitable solvent such as chloroform, dichloromethane, or
toluene. When the monosaccharide derivative in question is a
per-O-acylated glycosyl bromide or chloride, promotors such
as silver trifluoromethane sulphonate or mercury (II)salts
(H. Paulsen, *Angew. Chem. Int. Ed. Engl.*, 21 (1982) 155-173)
can be used and the reactions are carried out in a suitable
solvent such as dichloromethane or toluene. The glucose W_1R_1
or $W_1R_1PG_1$-glycosides are obtained after de-O-acylation using
sodium methoxide (A. Thompson, M. L. Wolfrom, and E. Pascu,
page 215-220, *Methods in Carbohydrate Chemistry*, Vol II,
Editors: R. L. Whistler and M. L., E. Wolfrom, Academic
Press, New York, 1963) in methanol or in methanol containing
a co-solvent such as dichlormethane or tetrahydrofurane.

The 4,6-(4'-methoxy)benzylidene acetals are then obtained by
reaction with 4-methoxybenzaldehyde dimethyl acetal and acid
in a polar non-protic solvent such as e.g. dimethyl formami-
de, acetonitrile or tetrahydrofurane (J.J. Patroni *et al.*,
Aust. J. Chem. 1988,(41),91-102; for other methods of acetal
formation, see for example A. N. de Belder, 1979, *adv. Carbo-
hydr. Chem. Biochem.*, 34, 179 and references cited therein).

The epoxides B1 or B2

B_1 B_2
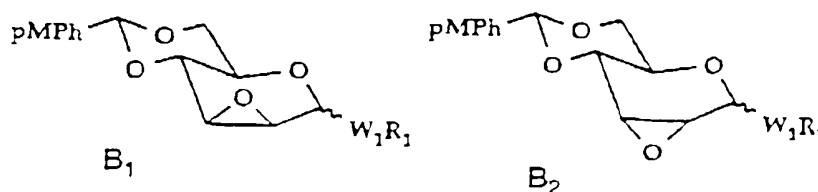
are then obtained through sulphonate esters: The *manno*
epoxides B1 can be prepared by reacting the glucoside deriva-
tive A with sodium hydride and p-toluenesulphonylimidazol in
dimethylformamide (D. Hicks and B. Fraser-Reid; *Synthesis*
1974, 203) or with sodium hydride and p-toluenesulphonyl
chloride in tetrahydrofurane (V.S. Murthy *et al*, *Synthetic
Commun.* 1993, 23(3), 285-289).

55

The *allo* epoxides B2 can be prepared by reacting the glucoside derivative A with methylsulphonyl or p-toluenesulphonyl chloride in pyridine and treating the resulting methylsulphonate diester with sodium ethoxide in ethanol (Y.
5 Ali, A. C. Richardson, *Carbohydrate Res.* 1967, 5, 441-448; N. Richtmeyer, *Methods in Carbohydrate Chemistry*, Vol 1,107).

The epoxides B1 or B2 can be reacted with suitable nucleophilic reagents to yield the diaxially substituted *allo* hexopyranosides C1 and C2
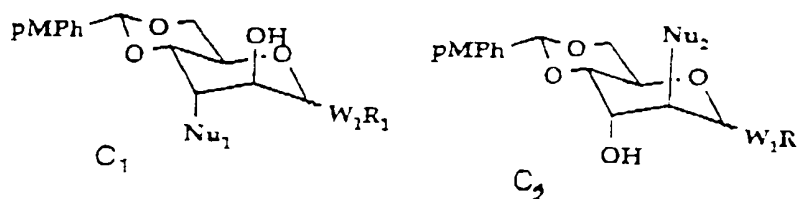
10 (for general references on the use of epoxides, see e.g.
J. Gorzynski Smith, *Synthesis*, 1984, 8, 629-656
Masamune S., Choy W., Petersen J S, and Sita L R, *Angew. Chem. Int. Ed. Engl.*, 1985, 24, 1-76; A. S. Rao et al., *Tetrahedron Lett.*, 1983, 39, 2323).

15 When the first atom of R_2 and Z_1 (as defined above) connected to the carbohydrate moiety in the desired final product is a nitrogen (=the nucleophilic atom), then the preferred nucleophile Nu_1 or Nu_2 is azide (N_3^-). The epoxide is treated with sodium azide and ammonium chloride in boiling 2-methoxy-
20 ethanol (R. D. Guthrie and D. Murphy; *J.Chem.Soc.* 1963, 5288-5294).

When the nucleophilic atom is oxygen or sulphur, the preferred general method of epoxide opening involves treatment with suitably protected alcohol or thiol in the presence of
25 neutral alumina in ether (G.H. Posner and D.Z. Rogers, *J. Am. Chem. Soc.* 1977, 99, 8208; G.H. Posner, D.Z. Rogers and A.

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Romero, *Isr. J. Chem.* 1979, 18, 259; and G.H. Posner, M. Hulce and R.K. Rose, *Synth. Commun.* 1981, 11, 737).

When the nucleophilic atom is carbon, the most commonly used reagents are organomagnesium, organolithium, organocopper,
5 organoaluminium and organoboron compounds (J. Gorzynski Smith, *Synthesis*, 1984, 8, 629-656 and references cited therein).

When the product is an *allo* hexopyranoside C1, the 2-hydroxy function can either be blocked with a protective group that
10 allows for the introduction of the R_2 functionality at a later stage (preferred if R_2 is an ester, not shown in figure), or the suitably protected, when necessary, functionality R_2 is introduced to produce D1

D_1
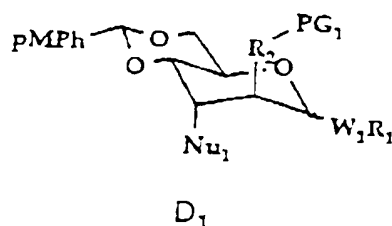
For example, OH-groups to ethers or esters (*Protective Groups*
15 *in Organic Synthesis*, Editors T. W. Greene and P. G. M. Wuts, John Wiley & Sons, Inc., New York, 1991); OH-groups to carbonates (J. March, *Advanced Organic Chemistry-Reaction Mechanisms, and Structure*, 3rd Edn. John Wiley & Sons, New York, 347 (1985), and references cited therein); OH-groups to
20 carbamates (J. March, *Advanced Organic Chemistry-Reaction Mechanisms, and Structure*, 3rd Edn. John Wiley & Sons, New York, 791-792 (1985), and references cited therein); OH-groups to alkylgroups via exomethylene derivatives and subsequent hydrogenation or via other routes (H. O. H. House,
25 *Modern Synthetic Reactions*, 2nd Edn. W. A Benjamin, Inc., Menlo Park, C.A., 1-130 (1972), and references cited therein; J. Yoshimura, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 69-134); and exchange of OH-groups for heterocyclic groups, via

different routes (A. R. Katritzky, *Handbook of Heterocyclic Chemistry*, Pergamon Press, Oxford, 1985).

When the product is an *allo* hexopyranoside C2, the 3-hydroxy function is blocked with a protective group that allows for the introduction of the Z_1-R_3 functionality at a later stage resulting in intermediates of the type D2

$$pMPh \diagdown O \diagdown O \diagdown Nu_2 \cdots O \diagdown W_1R_1, \quad OPG_2$$
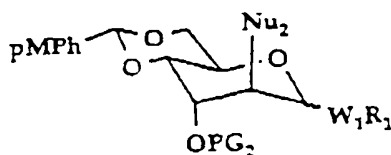
D₂

(*Protective Groups in Organic Synthesis*, Editors T. W. Greene and P. G. M. Wuts, John Wiley & Sons, Inc., New York, 1991) or transformed to a *manno* hexopyranoside intermediate D3

$$pMPh \diagdown O \diagdown O \diagdown Nu_2 \cdots O, \quad Nu_1 \diagdown W_1R_1$$

D₃
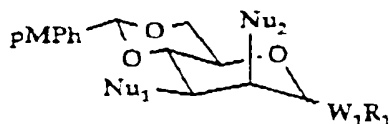
where Nu_1 is a protected or masked form of the functionality Z_1. OH-groups to ethers or esters (*Protective Groups in Organic Synthesis*, Editors T. W. Greene and P. G. M. Wuts, John Wiley & Sons, Inc., New York, 1991); OH-groups to azido-groups: J. March, *Advanced Organic Chemistry-Reaction Mechanisms, and Structure*, 3rd Edn. John Wiley & Sons, New York, 380, (1985), and references cited therein; H. H. Baer, *Pure Appl. Chem.*, 61(7) (1989) 1217-1234, and references cited therein; OH-groups to aminogroups via azides or other routes (March, *Advanced Organic Chemistry-Reaction Mechanisms, and Structure*, 3rd Edn. John Wiley & Sons, New York, 1106, 798-800 (1985), and references cited therein;

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H. H. Baer, *Pure Appl. Chem.*, 61(7) (1989) 1217-1234, and
references cited therein).

The 4,6-O-acetal function is then reductively opened to yield
either the function R_6 in the case when R_6 is an ether,

5   or the intermediates F1, F2 or F3 with a hydroxy function on
position 6 (reductive opening of acetals, see
Garegg P J and Hultberg H, *Carbohydr. Res.* 1981, 93, c10-11;
Garegg P J, Hultberg H, and Wallin S, *Carbohydr. Res.* 1982,
108, 97-101; Liptak A, Jodal I, Nanasi P, *Carbohydr. Res.*

10 1975, 44, 1-11; Baker D C, Horton D, Tindall C G, *Methods in
Carbohydr. Chem.*, 1976 Vol 6, 3-6; Mikami T, Asano H, Mitsu-
nobu O, *Chem. Lett.* 1987, 10, 2033-2036; Ek M, Garegg P J,
Hultberg H, Oscarsson S, *J. Carbohydr. Chem.* 1983,2, 305-311;
Hunter R, Bartels B, Michael J P, *Tetrahedron Lett.* 1991, 32,

15 1095-1098; Rao S P, Grindley T B, *Carbohydr. Res.* 1991, 218,
83-93; Hunter,R. Bartels,B. *J. Chem. Soc. Chem. Commun.* 1991,
2887-2888).

For example, the intermediates of type D1, D2 or D3 are
treated with sodium cyanoborohydride and chlorotrimethylsi-

20 lane in acetonitrile,
(R. Johansson and B.Samuelsson, *J. Chem. Soc. Perkin Trans.
I*, 1984, 2371-2374) or borane-trimethylamine complex and
aluminium trichloride. The regiochemical outcome of the
reaction is often solvent-dependent (Ek M, Garegg P J, Hult-

25 berg H, Oscarsson S, *J. Carbohydr. Chem.* 1983, 2, 305-311).

The aldehyde intermediates of type G1, G2 or G3

G1   G2   G3

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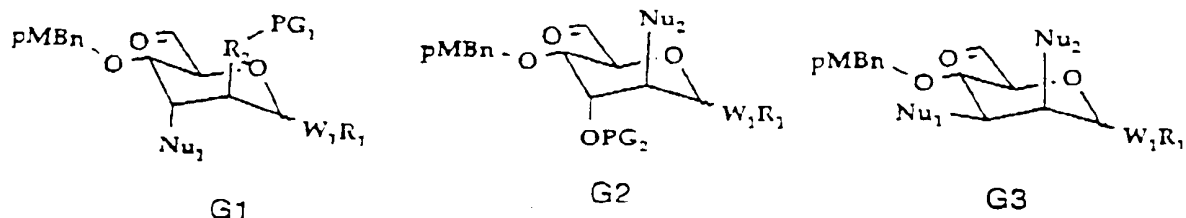
are obtained by oxidation of the corresponding 6-alcohol
intermediates of type D1, D2 or D3, preferrably by the Swern
procedure. (Mancuso A J, Swern D, *Synthesis*, 1981, 165-185;
Tidwell T, *Synthesis*, 1990, 857-870; for other oxidation
5 methods, see A. H. Haines, 1988, *Methods for the Oxidation of
Organic Compounds*, Chapter 2, Academic Press, San Diego, and
references cited therein).

In the next step a carbon nucleophile is added to the
aldehyde function of the intermediates of type G1, G2 or G3.
10 Preferrably, a suitably protected alkyllithium or aryllithium
reagent or a gringard reagent is added to the aldehyde in an
ether or hydrocarbon solvent to produce the secondary alco-
hols H1, H2 and H3:

H1   H2   H3

For reactions of aldehydes with organolithium and organomag-
15 nesium compounds, see J. March, *Advanced Organic Chemistry-
Reaction Mechanisms, and Structure*, 3rd Edn. John Wiley &
Sons, New York, 347 (1985), and references cited therein.
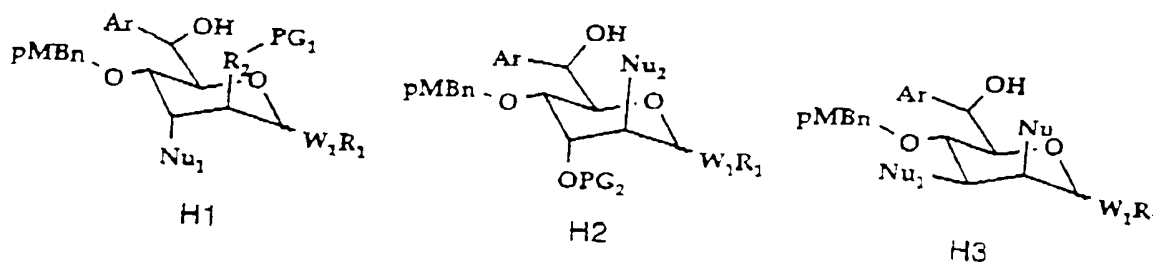
For reactions of aldehydes with organolithium and organomag-
nesium and other carbon nucleophils, see Evans D A, *Aldri-
20 chim. Acta*, 1982, 15, 23, and references cited therein.

For specific examples on the use and preparation of arylsub-
stituted phenyllithium and grignard reagents, see Ames M M,
Castagnoli Jr. N, *J. Labelled Compd.*, 1974,10(2), 195-205; DE
3807910 A1; Mills R J, Snieckus V, *Polynucl. Aromat. Hydro-
25 carbons, [Pap. Int. Symp.]*, 8th, Meeting 1983, 913-24. Edited
by: Cooke M and Dennis A J, Battelle Press 1985: Columbus,

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Ohio; Iriye R, Furukawa K, Nishida R, Kim C, Fukami H, *Biosci. Biotechnol. Biochem.* 1992,56(11), 1773-5; Comber M F, Sargent M V U, *J. Chem. Soc., Chem. Commun.*, 1991, (3), 190-2; Hirai T, Yoshizawa A, Nishiyama I, Fukumasa M, Shiratori N, Yokoyama A, EP 341686 A2; Leeson P D, Emmett J C, Shah V P, Showell G A, Novelli R, Prain H D, Benson M G, Ellis D, Pearce N J, Underwood A H, *J. Med. Chem.* 1989, 32(2), 320-36); Meltzer P C, Liang A Y, Brownell A L, Elmaleh D R, Madras B K, *J. Med. Chem.*, 36(7), 855-62; Willard A K, Novello F C, Hoffman W F, Cragoe Jr E J. US 4,459,423.

For substituted phenyllithium reagents that can be further elaborated into heterocyclic compounds, see: Lang H J, Muschaweck R, AU 514406 B2; Lang H J, Muschaweck R, Hropot M, HU 19761; Lang H J, Muschaweck R, Hropot M, DE 2737195.

For heteroaromatic aryllithiums and grignard reagents, see: Yang Y, Martin A R, Nelson D L, Regan J, *Heterocycles*, 1992, 34(6), 1169-75.

For examples of other organometallic reagents for the stereoselective synthesis of secondary alcohols from aldehydes:

Homoallyl alcohols with crotylmolybdenum complexes: Faller J W, John J A, Mazzieri M R, *Tetrahedron Lett.* 1989, 30, 1769-1772.

Homoallyl alcohols with titanium complexes: Riediker M, Duthaler R O, *Angew. Chem. Int. Ed. Engl.*, 1989, 28, 494-495.

3-Pyrollyl alcohols with silyl protected 3-lithiopyrrole: Bray B L, Mathies P H, Naef R, Solas D R, Tidwell T T, Artis D R, Muchowski J M, *J. Org. Chem.*, 1990, 55, 6317-6328.

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Allylic alcohols with E-vinylalane: A P Kozikowski and
Jiang-Ping Wu, *Tetrahedron Lett.* 1990, 30, 4309-4312 and
references cited therein.

Trans-allylic diols with vinylstannanes: Corey E J,
5   Wollenberg R H, *J. Org. Chem.*, 1975, 40, 2265-2266.

Pyrrolidine carbinols with α-lithio pyrrolidine amidines:
Sanner M A, *Tetrahedron Lett.* 1989, 30, 1909-1912.

Organozinc reagents: Fürstner A, *Synthesis*, 1989, 571-
590, and references cited therein.

10   Thereafter, the substituent on the 3-position (Nu_1 or OPG_1)
is transformed into a nucleophile in order to install the
negatively charged functionality Z_1-D-R_3.

The secondary 6-alcohols H1, H2 and H3 are first protected,
(*Protective Groups in Organic Synthesis*, Editors T. W. Greene
15   and P. G. M. Wuts, John Wiley & Sons, Inc., New York, 1991)
or transformed to amino functions in the cases where X_1-R_6
forms a peptide functionality (OH-groups to aminogroups via
azides or other routes: See for examples March, *Advanced
Organic Chemistry-Reaction Mechanisms, and Structure*, 3rd
20   Edn. John Wiley & Sons, New York, 1106, 798-800 (1985), and
references cited therein; H. H. Baer, *Pure Appl. Chem.*, 61(7)
(1989) 1217-1234, and references cited therein).

For peptide synthesis, see Gross and Meienhofer, *The Pep-
tides*, 3 vol., Academic Press, New York,1979-1981; Grant G A
25   *et al.*, *Synthetic Peptides, A Users Guide*, 1992, W. A. Free-
man and Company, New York, and references cited therein).

The 3-position is deprotected to an alcohol, thiol or amine
intermediate I1, I2 or I3
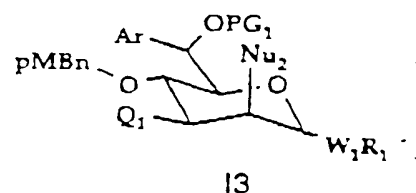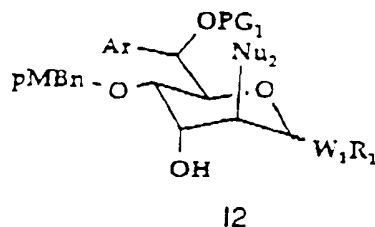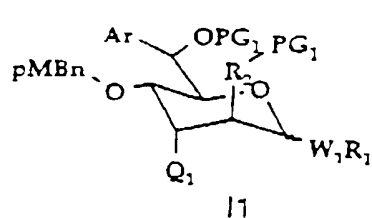
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$$I1 \qquad I2 \qquad I3$$

(*Protective Groups in Organic Synthesis*, Editors T. W. Greene
and P. G. M. Wuts, John Wiley & Sons, Inc., New York, 1991,
and references cited therein).

5 For example, treatment of I1, where R_2-PG$_1$ is a combination
of ether functions and Q_1 is azide, with gaseous hydrogen
sulphide in pyridine and water (T. Adashi, Y. Yamada, I.
Inoue and M. Saneyoshi, *Synthesis*, 1977, 45). For other
methods of azide reductions, see Poopeiko N E, Pricota T I,
Mikhailopulo I A, *Synlett*, 1991, 5, 342; Samano M C, Robins M
10 J, *Tetrahedron Lett*. 1991, 32, 6293-6296; Rakotomanomana N,
Lacombe J-M, Pavia A, *Carbohydr. Res.*, 1990, 197, 318-323;
Malik A A, Preston S B, Archibald T G, Cohen M P, Baum K,
Synthesis, 1989,450-451; Maiti S, Spevak P, Reddy N, *Synt.
Commun.* 1988, 18, 1201-1206; Bayley H, Standring D N, Knowles
15 J R, *Tetrahedron Lett.*, 1978, 39, 3633-3634).

The intermediates I1, I2 or I3 are sulphated with sulphur
trioxide-pyridine or -triethylamine complex to obtain the
intermediates J2, J4 and J6

$$J2 \qquad J4 \qquad J6$$
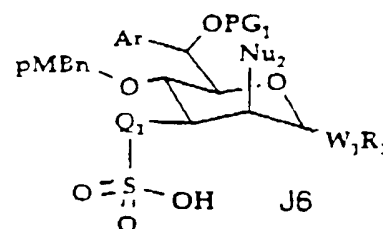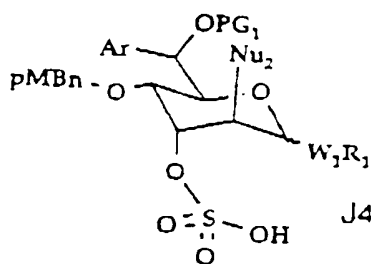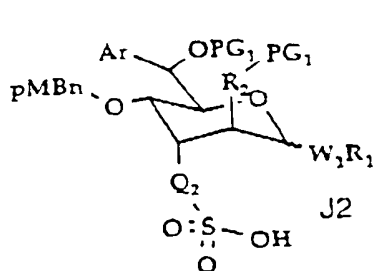
(see, for example J. Basten, G. Jaurand, B. Olde-Hanter, M.
Petitou and C.A.A. van Boeckel, *Bioorg. Med. Chem. Lett*,
1992, 2(9), 901-904 and references cited therein; J.Basten,
G. Jaurand, B. Olde-Hanter, P. Duchaussoy, M. Petitou and
5   C.A.A. van Boeckel, *Bioorg. Med. Chem. Lett*, 1992, 2(9), 905-
910, and references cited therein; Böcker,T. Lindhorst,TK.
Thiem,J. Vill,V. *Carbohydr. Res.* 1992, 230, 245-256) or
coupled to form the phosphoester intermediates J1, J3 and J5
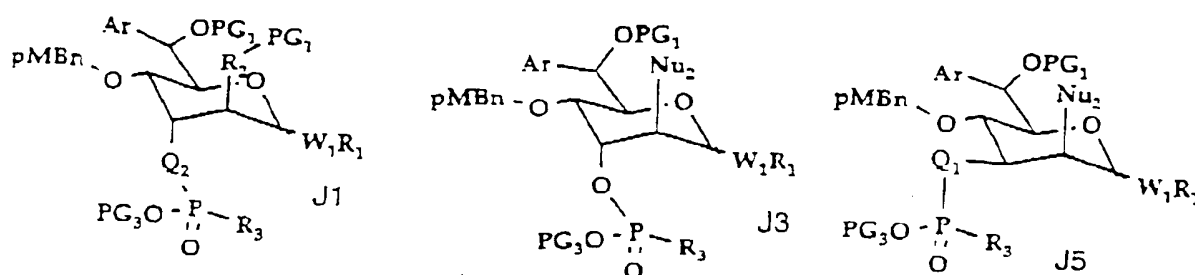
Since the phosphorus-nitrogen bond is known to be acid
10   labile, the intermediates leading to phosphodiester end-
products are preferred (M. Selim and T. N. Thanh, *C.R.
Seances Acad. Sci*, 1960, 250, 2377).

For example, the alcohol intermediates I1, I2 or I3 are
treated with 2,2,2-trichloroethyl 2-chlorophenyl phosphochlo-
15   ridate in chloroform and pyridine to form the phosphate
triesters. The 2,2,2-trichloroethyl group is removed by
treatment with zinc powder and the resulting phosphate dies-
ter is activated with 3-nitro-1-(2,4,6-triisopropylbenzene-
sulphonyl)-1,2,4-triazol e and coupled with the alcohol R₃-OH
20   to form the intermediates J1, J3 and J5. The 2-chlorophenyl
group is removed by treatment with pyridine-2-aldoxime and
N,N,N,N-tetramethylguanidine in moist pyridine (see for
example P. J. Garegg, R. Johansson, I. Lindh and B. Samuels-
son, *Carbohydr. Res.* 1986, 150, 285-289).

25   Alternatively, by the phosphite triester approach, alcohol
intermediates I1, I2 or I3 are treated with phenyl chloro-
N,N-diisopropylphosphoramidite in acetonitrile to form carbo-

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hydrate phosphoamidites. After purification by chromatography, these are exposed to an alcohol R_3-OH in the presence of a mild acid, such as pyridinium p-toluenesulphonate and treated with tert-butyl hydroperoxide to form phosphate

5   diesters J1, J3 and J5 (PG$_3$ = H) (H-N. Caro, M. Martín-Lomas and M. Bernabé, *Carbohydr. Res.* 1993, 240, 119-131, and references cited therein).

For a review on phospodiesters in DNA synthesis, see Narang S, *Tetrahedron*, 1983, 39, 1-22 and D. W. Hutchinson, 1991,

10 Chapter 3 in *Chemistry of Nucleosides and Nucleotides*, vol 2, ed. B. Townsend, Plenum Press, New York, and references cited therein. For other examples on carbohydrate phosphodiester synthesis, see for example Ichikawa Y, Sim M M, Wong C H, *J. Org. Chem.* 1992, 57, 2943-2946 and Schmidt R R, Braun H, Jung

15 K-H, *Tetrahedron Lett.* 1992, 33, 1585-1588. For synthesis of modified phosphodiester linkages, See R. S. Varma, 1993, *Synlett,* 621-636, and references cited therein.

To obtain arylphosphonic acid esters and amides J1, J3 and J5, where R_3 is an alkyl or aromatic group the arylphosphonic

20 acid R_3-PO$_3$H$_2$ is coupled to the alcohol intermediates I1, I2 or I3, for example, with a carbodiimide reagent, or treatment of the phosphonic dichlorides with the alcohol intermediates I1, I2 or I3 in pyridine (T. H. Siddal III, and C. A. Prohaska, *J. Am. Chem.* 1962, 84, 3467).

25 The alkylphosphonic triesters are formed from trialkyphosphites and alkyl halides by the Arbuzov reaction (Arbuzov, *Pure Appl. Chem.* 1964, 9, 307-335, J. March, *Advanced Organic Chemistry-Reaction Mechanisms, and Structure,* 3rd Edn. John Wiley & Sons, New York, 347 (1985), and references cited

30 therein).

For the preparation of arylphosphonic triesters via phosphorus trichloride, see for example: K. Sasse, *Methoden der Organichen Chemie* (Houben-Weyl), 4th ed., Vol. 12/1, Georg Thieme, Stuttgart, 1963, p. 314 and p. 392 and references

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cited therein; G. M. Kosolapoff, *Org. React.* 6, 273 (1951), and references cited therein; L.D. Freedman and G. O. Doak, *Chem. Rev.* 1957, 57, 479, and references cited therein.

5   For the preparation of arylphosphonic triesters via organo-magnesium or organolithium reagents, see for example: K. Sasse, *Methoden der Organichen Chemie* (Houben-Weyl), 4th ed., Vol. 12/1, Georg Thieme, Stuttgart, 1963, p. 372, and references cited therein; G. M. Kosolapoff, *Org. React.* 6, 273 (1951), and references cited therein.

10   Intermediates J1, J2, J3, J4, J5 and J6 are deprotected to form the final products (*Protective Groups in Organic Synthesis*, Editors T. W. Greene and P. G. M. Wuts, John Wiley & Sons, Inc., New York, 1991, and references cited therein) and transformed to their sodium or potassium salts.

15   It is justified to assume that the compounds described above are capable of interacting with sites in *PapD* and other periplasmic chaperones. In order to establish that this is really the case, assays like those described in the examples should be performed.

20   Thus, a preferred compound of the invention is a compound as described above, which causes a prevention, inhibition or enhancement of the binding of G1'-19'WT to *PapD*, and/or causes a prevention, inhibition or enhancement of the binding of the fusion peptide MBP-G1'-140' to *PapD* and/or causes a
25   prevention, inhibition or enhancement of the binding of the peptide G125'-140' (which have the sequence SEQ ID NO: 20) to *PapD* and/or is capable of inhibiting the restoration of the *PapD-PapG* complex normally caused by the addition of access *PapD*.

30   The assay used to establish that a substance exhibits one of the above effects is preferably one of the assays described in the examples herein. Of course, also other assays as those discussed above in the methods of the invention may be uti-

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lized in order to establish that the compound actually is
capable of inhibiting pilus assembly.

One important point which should be taken into consideration
when setting up an assay, is the role the chaperones are
5   playing *in vivo*. They bind to the pilus subunits already
during the transport through the cell membrane of the sub-
units and it is therefore assumed, that the pilus subunits
are more or less unfolded (i.e. not in their final folded
conformation) when they bind to the chaperone. It has been
10   observed by the inventors that the kinetics of binding
between completely folded pilus subunits (or analogues there-
of) and the chaperone *PapD* is a slow process, although it is
known that the process of pilus assembly is relatively fast
in vivo. In order to speed up the rate of assembly of the
15   chaperone/pilus subunit complex *in vitro* it is contemplated
that more or less severe denaturing conditions could be
imposed on the pilus subunits (or the analogues thereof)
prior to the assay. Such denaturing could be obtained by
subjecting the pilus subunits to physical stress (e.g. to
20   elevated temperature, pressure changes, radiation etc.) or to
changes in the chemical environment (e.g. changes in ionic
strength, changes in pH, or the addition of denaturing com-
pounds or disulphide reducing compounds.

The expression "denaturing compound" refers to a compound
25   which when present as one of the solutes in a liquid phase
comprising polypeptide molecules may destabilize folded
states of the polypeptide molecules leading to partial or
complete unfolding of the polypeptide chains. The denaturing
effect exerted by a denaturing compound increases with in-
30   creasing concentration of the denaturing compound in the
solution, but may furthermore be enhanced or moderated due to
the presence of other solutes in the solution, or by changes
in physical parameters, e.g. temperature or pressure.

As examples of suitable denaturing compounds to be used may
be mentioned urea, guanidine-HCl, di-C_{1-6}alkylformamides such
as dimethylformamide and di-C_{1-6}-alkylsulphones.

Examples of disulphide reducing compounds are glutathion-
yl-2-thiopyridyl disulphide, 2-thiocholyl-2-thiopyridyl
disulphide, 2-mercaptoethanol-2-thiopyridyl disulphide and
mercaptoacetate-2-thiopyridyl disulphide.

A set of observations confirm the assumption that the pilus
subunits are more or less unfolded while bound to the chape-
rone: As described in Kuehn et al., 1991, it is possible to
restore the PapD-PapG complex after denaturation by adding
access PapD. Further, preliminary results obtained by capil-
lary electrophoresis show that denaturation of the fusion
protein MBP-G1'-140' gives a single form of the fusion pro-
tein, whereas two forms of the fusion protein are observed in
capillary electrophoresis before denaturation, one major
which is unable to interact with PapD and one minor which is
capable of interacting with PapD. It is therefore contem-
plated that the denatured fusion protein may serve as a
superior substrate in the different competitive assays
described herein as does the non-denatured form of the fusion
protein.

Thus, in a preferred embodiment of the invention, the com-
pound of the invention causes a prevention, inhibition or
enhancement of the binding of a denatured form of either a
pilus subunit or an analogue thereof to PapD, and/or causes a
prevention, inhibition or enhancement of the binding of a
denatured form of MBP-G1'-140' to PapD and/or causes a pre-
vention, inhibition or enhancement of the binding of a dena-
tured form of G125'-140' to PapD.

As will appear from the examples, compounds which should be
capable of interacting with PapD and other chaperones have
already been identified and synthesized. These compounds

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which are all pyranosides are also an important part of the
invention:

Ethyl 2,3-O-Dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyrano-
side;

5   Ethyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-gluco-
hexopyranoside;
Methylglycolyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-β-D-glu-
cohexopyranoside;
2-(Hydroxy)ethyl 4-O-benzyl-β-D-glucopyranoside;

10   Sodium glycolyl 4-O-benzyl-β-D-glucohexopyranoside;
Methyl 2-O-ethyl-4,6-O-(4'-methoxy)phenylmethylene-α-D-manno-
hexopyranoside;
Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,6-O-(4'-me-
thoxy)phenylmethylene-α-D-mannohexopyranoside;

15   Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-methoxy)-
benzyl-α-D-mannohexopyranoside;
methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-6-O-(4'-methoxy)-
benzyl-α-D-mannohexopyranoside;
Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-methoxy)-

20   benzyl-6(S)-phenyl-α-D-mannohexopyranoside;
Methyl 2,3-anhydro-4,6-O-p-methoxybenzylidene-α-D-mannopyra-
noside;
Methyl 3-azido-4,6-O-p-methoxybenzylidene-α-D-altropyrano-
side;

25   Methyl 3-azido-2-O-ethyl-4,6-O-p-methoxybenzylidene-α-D-
altropyranoside;
Methyl 3-azido-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-α-D-
altro-pyranosid;
Methyl 3-azido-6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxy-

30   benzyl-α-D-altropyranoside;
Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
sulfamino-α-D-altropyranoside sodium salt;
Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-3-sulfamino-α-D-altropy-
ranoside ammonium salt;

35   Methyl 3-azido-6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxy-
benzyl-α-D-altropyranoside;

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Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
sulfamino-α-D-altropyranoside sodium salt;
Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-3-sulfamino-α-D-altro-
pyranoside ammonium salt;
5   Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
tbutyloxamido-α-D-altropyranoside;
Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-3-oxamido-α-D-altro-
pyranoside ammonium salt;
Methyl 3-azido-6-O-pyrrol-3'-ylcarboxyl-3-deoxy-2-O-ethyl-4-
10   O-p-methoxybenzyl-α-D-altropyranoside; and
Methyl 6-O-pyrrol-3'-ylcarboxyl-3-deoxy-2-O-ethyl-3-sulfami-
no-α-D-altropyranoside ammonium salt.

Other compounds are of course also possible as interactors
with sites in chaperones. As is evident from example 7,
15   modified peptides also may prove to be useful in the methods
of the invention.

As will be clear from the above, the identification of a site
in the chaperone which may be affected so as to interfere
with pilus assembly is a critical starting point in the
20   methods described herein for the identification, isolation
and synthesis of compounds capable of interacting with peri-
plasmic chaperones.

Thus, the invention also relates to a method for identifying
a binding site in a molecular chaperone, comprising

25   co-crystallizing the periplasmic molecular chaperone or
          an analogue thereof with a ligand binding to the peri-
          plasmic molecular chaperone or the analogue thereof,

          resolving the three-dimensional structure of the chapero-
          ne/ligand interaction, thereby resolving the three-dimen-
30   sional structure of the periplasmic molecular chaperone
          or the analogue thereof when binding to the ligand,

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determining the site-point(s) involved in the
intermolecular interaction between the periplasmic mole-
cular chaperone or the analogue thereof and the ligand,
and

5   identifying the thus determined site-point(s) of the
periplasmic molecular chaperone or the analogue thereof
as a binding site in the periplasmic molecular chaperone
or the analogue thereof.

By the term "ligand" as used herein, is meant a substance
10 which exhibit binding to a host or receptor molecule (in this
connection a chaperone). The binding is not a non-specific
interaction, which means that a binding motif between the
ligand and the host or receptor molecule exists. In other
words, when bringing a sample of the ligand and a sample of
15 the host or receptor molecule in contact with each other the
complexes formed between the ligand and the host or receptor
molecule will substantially all reflect the same
intermolecular interactions.

As mentioned above one embodiment of the invention is to
20 administer a substance which is capable of preventing, inhib-
iting or enhancing binding between pilus subunits and molecu-
lar chaperones.

Accordingly the invention relates to a pharmaceutical compo-
sition, comprising, as an active compound, a substance
25 capable of interacting with at least one type of periplasmic
molecular chaperone which binds pilus subunits during trans-
port of these pilus subunits through the periplasmic space
and/or during the process of assembly of the intact pilus, in
such a manner that binding of pilus subunits to the periplas-
30 mic molecular chaperone is prevented, inhibited or enhanced,
in combination with at least one pharmaceutically acceptable
carrier or excipient. Preferably such a substance is a sub-
stance according to the invention or a substance iden-
tified/designed according to the methods of the invention.

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The pharmaceuticals and pharmaceuticals discussed herein are
thus, according to the invention, for the treatment and/or
prophylaxis of the same conditions as those discussed when
disclosing the method of treatment of the invention and
5 caused by the same bacterial species.

Therefore, a pharmaceutical composition, comprising, as an
active compound, a substance used in the therapeutic methods
of the invention, a substance according to the invention or a
substance identified according to the methods of the inven-
10 tion, in combination with at least one pharmaceutically
acceptable carrier or excipient, is a part of the invention.

Such pharmaceutical compositions of the invention could also
comprise at least one additional pharmaceutical substance,
which i.a. could enhance the pharmaceutical effects exerted
15 by the substance of the invention or the substance identified
according to the invention.

Additional pharmaceutical substances could be steroid hor-
mones, disinfectants, anti-pyrretics, etc. Preferably such an
additional substance could be an antibacterial agent.

20 Such an anti-bacterial agent could conveniently be selected
from the group consisting of penicillins, cephalosporins,
aminoglycosides, sulfonamides, tetracyclines,
chloramphenicol, polymyxins, antimycobacterial drugs and
urinary antiseptics.

25 The invention also relates to a substance employed in the
methods of the invention as well as a substance of the inven-
tion and a substance identified according to the methods of
the invention for use as a pharmaceutical. It is preferred
that the pharmaceutical is for anti-bacterial treatment
30 and/or prophylaxis, especially treatment and/or prophylaxis
of diseases caused by tissue-adhering pilus forming bacteria,
and it is especially preferred that the pharmaceutical is for
treatment and/or phrophylaxis of urinary tract infection.

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Finally, the invention relates to the use of a substance
employed in the methods of the invention as well as a sub-
stance of the invention and of a substance identified accor-
ding to the methods of the invention for the preparation of a
5 pharmaceutical composition for the treatment and/or prophy-
laxis of bacterial infection.

LEGENDS TO FIGURES

Fig. 1:   PapD binds to pilus subunit-related peptides coated
          in ELISA microtiter plates and in solution. The
10   sequences of the purified, synthetic peptides used
          in this study are described separately in Flemmer
          K, Walse B, Roy S, and Kihlberg J, manuscript in
          preparation. A and B: The peptides from table 1
          were coated on microtiter wells and tested for
15   their ability to bind to PapD in an ELISA assay
          described in example 2. Water insoluble peptides
          were dissolved in 2.5% acetic acid (AA) which had
          no effect on the binding of G1'-19'WT to PapD in
          this assay. Each graph represents the average of
20   duplicate wells.

Fig. 2:   Binding of pilus subunit-related peptides provides
          protection against enzymatic proteolysis and blocks
          binding of PapD to PapG.
          A Upper: PapD (15 μg) was incubated with PBS (lane
25   D) or with 1.5 μg trypsin (lane D and Tr) at 37°C
          for 20 minutes and applied to 20% SDS-PAGE. Coomas-
          sie blue stained bands corresponding to full length
          PapD (PapD), trypsin (Tr, the NH_2-terminal fragment
          of PapD (N), and the COOH-terminal fragment of PapD
30   starting at residue 100 (C) are indicated).
          A Lower: The rate of trypsin cleavage of PapD
          decreased upon preincubation with the G1'-19'WT,
          G1'-16'WT or K1'-19'WT peptides, but the G2'-21'a-
          mide peptide had no effect. 50 μg of purified PapD
35   was preincubated for 15 min at 25°C with a 20-fold

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molar excess of G1'-19'WT, G1'-16'WT, K1'-19'WT or
G2'-21'amide peptides (any trypsin cleavage sites
in these peptides occur at their amino termini
where they were predicted not to interfere with
PapD), or an equivalent volume of water. Each
sample was then incubated at 37°C with 3.2 μg of
trypsin. Aliquots were removed after 0, 5, 10, 20,
30 and 40 min and boiled in SDS-PAGE sample buffer
to stop the digestion. The samples were applied to
15% SDS-PAGE gels and the amount of full-length
PapD remaining was determined by densitometry of
Coomassie blue stained bands corresponding to full-
length *PapD* which are shown.

B: *PapD* incubated with the G1'-19'WT, G1'-16'WT,
K1'-19'WT, and G2'-21'amide was added to reduced,
denatured *PapD-PapG* and the amount of *PapD-PapG*
complex restored in each sample was quantitated as
described in example 2. The % inhibition represents
the amount of the *PapD-PapG* complex restored with
peptide-treated *PapD* compared to the amount of
PapD-PapG complex restored with untreated *PapD*. The
graph represent the average of 4 experiments. *PapD*
and *PapD-PapG* complex were prepared as described in
F. Lindberg et al, *J. Bacteriol.* 171, 6052 (1989)
and S.J. Hultgren et al., *Proc. Natl. Acad. Sci.*
USA 86, 4357 (1989).

Fig. 3   Stereoscopic view of the three-dimensional struc-
          ture of PapD co-crystallized with the G1-19WT
          peptide determined to 3.0 Å resolution. The peptide
          is bound in an extended conformation along the G1 β
          strand in the cleft of PapD with the terminal car-
          boxylate group forming hydrogen bonds with residues
          Arg-8 and Lys-112 of PapD.

Fig. 4   Stereoscopic view of the 3.0 Å resolution electron
          density of the G1-19WT peptide COOH-terminus and
          neighbouring PapD residues, superimposed on the

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refined structure. The electron density map was
calculated with coefficients (2|Fo|-|Fc|) and con-
toured at 1σ.

Fig. 5   Stereoscopic view of a PapD-peptide complex showing
5   its interaction with a twofold non-
          crystallographically related complex.

Fig. 6   Superposition of COOH-terminal domains of native
          PapD (thick lines) and complexed PapD (thin lines)
          showing the 13° difference in the hinge bend angle
10   between the two structures. The structures were
          superimposed using the LSQ option the program O.
          The resulting rms for 98 Ca atoms of the COOH ter-
          minal domain was 0.66 Å.

Fig. 7:   Ability of PapD Arg-8, Lys-112 and Thr-7 mutants to
15   bind PapG and restore the PapD-PapG complex in
          vitro. 0.4 μg of PapD-PapG complex was reduced and
          denatured as described in Fig. 2B. The denatured
          PapD-PapG complex was then diluted with 0-630 ng
          purified wild type (WT) or mutant PapD. The amount
20   of PapD-PapG restored in the samples was determined
          as in Fig. 2B and was graphed as a percentage of
          the greatest amount of PapD-PapG restored. The
          values graphed represent the average of 3 experi-
          ments. Wild type and mutant PapD and the PapD-PapG
25   complex were purified as described in F. Lindberg
          et al, J. Bacteriol. 171, 6052 (1989) and S.J.
          Hultgren et al., Proc. Natl. Acad. Sci. USA 86,
          4357 (1989).

Fig. 8:   6-hydroxydopamine, hdo_0, and other members of the
30   hdo family. hdo_0 Was used as starting-point for
          the construction of a family of compounds intended
          to bind to the binding-site of PapD. hdo_4: "Aryl"
          denotes 3,4-dihydroxyphenyl.
          hdo_6: "Aryl" is a 3,4-dihydroxyphenyl group.

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hdo_7: "Aryl" is a 3-hydroxyphenyl group.
hdo_8: "Aryl" is a 3-nitrophenyl group.
hdo_9: "Aryl" is a phenyl group.

Fig. 9: The hdo family and its interaction with PapD.
5   A: "Aryl" is a phenyl group, a heteroaromatic ring,
         or a substituted phenyl group with polar substitu-
         ents unsymmetrically in order to obtain hydrophobic
         contact with the protein on one side and interac-
         tion with solvent water on the other.
10   B: Examples of "Aryl" wherein R is hydrogen or
         alkyl; "X" is an oxygen atom, a sulphur atom, or a
         amino group; and CG" is a negatively charged group,
         such as a carboxyl group, a tetrazoyl group, a
         phosphate group, a phosphate ester or a sulphate
15   group.
         C: Examples of the group CG.

Fig. 10: General reaction scheme for the production of vari-
          ous members of the hdo family.

Fig. 11: General reaction scheme for the production of vari-
20   ous members of the bpy family.

Fig. 12: General reaction scheme for the production of vari-
          ous members of the bpy family.

Fig. 13: General reaction scheme for the production of vari-
          ous members of the bpy family.

25 Fig. 14: General reaction scheme for the production of vari-
          ous members of the bpy family.

Fig. 15: General reaction scheme for the production of va-
          rious members of the bpy family.

Fig. 16: General reaction scheme for the production of va-
30   rious members of the bpy family.

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Fig. 17: Graph over the inhibitory effect of different
          lengths of C-terminal peptide fragments of PapG on
          the binding between PapD and the fusion protein
          MBP-G1'-140'.

5   The G1'-8' fragment exhibits a significantly higher
          inhibitive effect on the binding than does the
          shorter G1'-6' and G1'-7' fragments.

Fig. 18: Graph over the inhibitory effect of different ana-
          logues of G1'-8' (wherein the 8 amino acid residues
10   one at a time has been replaced by serine (S) or
          Alanine (A)) on the binding between PapD and the
          fusion protein MBP-G1'-140'.
          The effect of the replacements of residues 4', 5'
          and 6' in G1'-8' reveals that these residues are
15   important in the interaction with PapD, as the
          replacement of these residues results in less inhi-
          bitory peptides.

Fig. 19: Graph over the inhibitory effect of different ana-
          logues of G1'-8' (wherein the 8 amino acid residues
20   one at a time has been deleted simultaneously with
          the addition of an N-terminal serine) on the bin-
          ding between PapD and the fusion protein MBP-
          G1'-140'.
          Also in this experiment, the significance of amino
25   acid residues 4', 5', and 6' are emphasized, as the
          deletions at these positions leads to less inhibi-
          tory peptides.

Fig. 20: MBP/G fusion constructs, PapG-truncates and syn-
          thetic peptides used in example 10.
30   The open box indicates the primary sequence of
          PapG. The positions of the four Cys residues are
          shown. The hatched bar represents MBP. The starting
          and ending residues of PapG fused onto the COOH-
          terminus of MBP are noted for each fusion. The
35   terminating residue for each PapG-truncate is also

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indicated. The names of the MBP/G fusion proteins
are listed. In the lower portion of the figure, the
solid boxes localize the PapD interactive sites and
the sequences of the four peptides used in the
example are listed (cf. also SEQ ID NOS: 19-22).

Fig. 21: PapD-MBP/G interactions in vivo.
Periplasmic extracts containing PapD and each MBP/G
fusion were subjected to amylose affinity chroma-
tography. The eluates were analyzed on A) a 12.5%
Coomassie blue stained SDS polyacrylamide gel; B) a
western blot using anti-PapD antiserum; or C) a
silver stained IEF gel. In Figs. 21A, B and C,
samples were purified form periplasmic extracts
containing PapD and MBP (lane 1), PapD and
MBP-G1'-19' (lane 2), PapD and MBP-G1'-81' (lane
3), PapD and MBP-G1'-140' (lane 4). The position of
co-purified PapD is indicated. MBP alone and MBP/G
fusion truncates also co-purified with the MBP/G
fusions. The molecular weight of the slowest migra-
ting bands on SDS-PAGE correspond to each full
length MBP/G fusion protein. On the IE F gel (C)
several bands could be seen for the same reasons. A
unique band at pI 5.2 was detected in Fig. 2C, lane
4. This band was excised, boiled in SDS sample
buffer and analyzed by western blotting with anti-
PapD and anti-MBP antisera. It was composed of both
MBP-G1'-140' and PapD (Fig. 2D, lane 1).

Fig. 22: Inhibition of chaperone function by expression of
MBP/G fusions in vivo.
Strains carrying pFJ22 (papDJKEFGA) and plasmids
encoding the MBP/G fusions were induced with 1 mM
IPTG. Periplasmic extracts containing pilus sub-
units and MBP (lane 1), or MBP-G1'-19' (lane 2), or
MBP-G1'-81' (lane 3), or MBP-G1'-140' (lane 4) were
analyzed by western blotting using anti-PapA
antiserum (A), or anti-PapD-PapG antiserum (B), or

anti-tip fibrillum antiserum (C). In this assay the
presence of the subunit indicates that chaperone-
subunit interactions occurred since subunits are
degraded in the absence of an interaction with the

5   chaperone. Note that the presence of the PapA, PapG
and PapF subunits decrease significantly when co-
expressed with MPB-G1'-140'.

Fig. 23:   Binding of PapD to MBP/G fusion proteins in vitro.
          (A) PapD was incubated with 1 μg of amylose affin-

10   ity purified MBP (lane), MBP-G1'-19' (lane 2),
          MBP-G1'-81' (lane 3), or MBP-G1'-140' (lane 4) and
          complex formation was evaluated on the silver
          stained IEF gel. Positions of MBP/G fusions, PapD
          and the PapD-MBP-G1'-140' complex are indicated.

15   (B) Amylose affinity purified MBP/G fusion proteins
          were coated to wells of microtiter plates. The
          concentration of the MBP/G proteins in the wells is
          indicated. Binding of 50 pmol/50 ml of PapD to the
          immobilized proteins was determined by ELISA using

20   anti-PapD antiserum.

Fig. 24:   Identification of PapD-PapG2 truncate complex by
          acidic native gel electrophoresis.
          Periplasm containing PapD and PapG, PapG2 or PapG3
          (lane 2, 3 and 4 respectively) were subjected to

25   Gal α(1-4) Gal chromatography and eth eluates were
          analyzed on acidic native gel electrophoresis fol-
          lowing western blotting using anti-PapD (A) and
          anti-PapG (B) antisera. Purified PapD-PapG complex
          was loaded in lane 1.

30 Fig. 25:   Characterization of a second site on PapG recog-
          nized by PapD.
          Four synthetic peptides overlapping the PapG region
          from residue 156' to 120' (indicated in Fig. 20)
          were coated to wells of microtiter plates. The

35   concentration of each peptide in the wells is indi-

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cated. Binding of 200 pmol/ 50 ml of *PapD* to the
immobilized peptide was determined by ELISA using
anti-*PapD* antiserum.

EXAMPLES

5   EXAMPLE 1
*Identification of the motif of binding between PapD and
G1'-19'WT*

Materials and Methods

PapD was prepared as described by A. Holmgren *et al.* in *J.*
10   *Mol. Biol.* 203, 279 (1988) and obtained from the Department
of Molecular Microbiology, Washington University School of
Medicine, St. Louis, USA.

The peptide G1'-19'WT was prepared using Fmoc solid phase
synthesis followed by purification by reversed phase HPLC.
15   The peptide was obtained form Department of Chemistry, Uni-
versity of Lund, Lund, Sweden. Table 1 indicates the peptides
used in binding assays in this application.

In the present application, amino acid residues or peptide
fragments originating from other peptides than *PapD* (e.g.
20   from *PapG* or from *PapK*) will be indicated with ', e.g. "G1'-
19'WT" or "Pro-1'", in order to distinguish such residues
from amino acid residues of *PapD*. Further, the numbering is
from the C-terminal end of the non-*PapD* peptide, *i.e.* G1'-19'
denotes the 19 C-terminal amino acid residues of *PapG*.

25   *Crystallisation of PapD-peptide complex*

Crystals of the *PapD*-peptide complex were obtained by vapour
diffusion against 20% PEG8000, 0.1 M cacodylate buffer at pH
5.0, and 0.2 M calcium acetate. The crystallisation drop
contained equal volumes of reservoir and protein solutions.
30   The protein solution (17 mg/ml) contained a 1:1 molar ratio

of PapD and peptide in 20 mM MES (2-[N-Morpholino]ethane sulphonic acid) at pH 6.5 with 1.0% β-octyl glucoside.

X-ray crystallography

The obtained crystals of the complex between PapD and the
5   protein were mounted inside sealed quartz-glass capillary
    tubes and initially characterised by examining them on a X-
    ray precession camera thereby obtaining pictures of the X-ray
    diffraction pattern on photographic film representing spa-
    tially non-distorted images of the reciprocal lattice of the
10 crystals. Using standard analysis of the images, it was
    determined that the crystals have a monoclinic space group,
    C2, with cell dimensions: a = 130.7 Å, b = 83.5 Å, c = 59.2
    Å, and β = 117.2°, two molecules in the asymmetric unit and
    diffract to 2.9 Å resolution on a lab X-ray source with a
15 rotating anode and Cu K_α target.

Collection and processing of experimental data

In order to solve the atomic structure of molecules by X-ray
crystallography, the positions and intensities of the dif-
fraction maxima have to be measured. Intensity data for the
20 PapD-peptide crystals were collected on a Huong-Hamlin multi-
    wire area-detector system (Xuong et al., 1985). All data were
    obtained from a single crystal and initially processing was
    carried out using the MADNES software package (Messerschmit
    and Pflugrath, 1987). Merging and scaling of the data was
25 carried out using ROTAVATA and AGROVATA from the CCP4 package
    (see CCP4, The SERC (UK) Collaborative Project No. 4, A Suite
    of Programs for Protein Crystallography, Darebury Laboratory,
    UK). The final data set contained 9592 independent reflec-
    tions with an Rsym of 6.8% for data between 20.0 and 3.0 Å

30 resolution (Rsym = $\sum \sum \frac{(|I_{hi}| - |I_h|)}{\sum_{hi} I_{hi}}$, where I_{hi} and I_h are the

intensities of the individual and mean structure factors,
respectively).

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Solution of three-dimensional structure

The structure of the *PapD*-peptide complex was solved using
the program XPLOR performing the standard method of molecular
replacement. The search model used was the refined 2.0 Å
5   resolution structure of *PapD*. Using 8.0 Å to 4.0 Å resolution
data, the self-rotation function gave a clear non-crystallo-
graphic two-fold axis, and the top two peaks of the cross-
rotation gave the correct solution which was improved using
Patterson correlation (PC) refinement. The correct solution
10 was also obtained from the top peaks in the translation
functions, the R-factor being 39.0% for 8.0 to 4.0 Å reso-
lution data (the R-factor defined as $R\text{-}factor = \dfrac{\sum |Fo| - |Fc|}{\sum Fo}$).

Refinement was obtained with subsequent rigid body refinement
in which all four domains of the 2 *PapD* molecules in the
15 asymmetric unit were allowed to refine independently resul-
ting in a R-factor of 36.4% for the same data.

Examination of an |Fo|-|Fc| electron density map at this
stage using the graphics program O showed clear density
corresponding to the peptide in the *PapD* cleft and running
20 along the surface of the protein. The orientation of the
peptide was easily determined from the electron density, but
initially only the final 10 C-terminal amino-acids of the
peptide could be modelled into density.

Refinement and analysis of structure.

25 Simulated annealing refinement with XPLOR was initiated at
this stage. Several additional cycles of model building and
refinement were carried out with a further 4 peptide amino-
acids being added to yield an R-factor for the current model
of 18.2% for 8.0 to 3.0 Å resolution data. The model at the
30 present stage of refinement (which contains no water mole-
cules and does not include the first 5 N-terminal amino-acids
of the peptide) has root-mean square (rms) deviations from

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ideal geometry of 0.020 for bonds lengths and 4.2° for bond
angles.

The peptide is seen to bind in an extended conformation with
the C-terminal pro-1' anchored within the inter-domain cleft
5   and proposed (6) subunit binding site. Hydrogen bonds are
formed between the peptide carboxy terminus and two invariant
positively charged residues of PapD, Arg-8 and Lys-112. Site-
directed mutagenesis has now confirmed that Arg-8 and Lys-112
are essential for the binding of pilin subunits both in vitro
10   and in vivo (cf. example 2)). The Pro-1' side chain makes van
der Waals contacts in the cleft with residues form both
domains of PapD: Thr-7, Thr-152, Ile-154, Thr-170 and Ile-
194. The neighbouring peptide residue, Phe-2', lies in a
shallow pocket formed between the two β-sheets of the N-
15   terminal domain and makes hydrophobic interactions with Leu-
4, Thr-7, Thr-109 and Ile-111. The peptide then runs along
the surface of the N-terminal domain, forming a parallel β-
strand interaction with strand G1. In this way 7 main chain
hydrogen bonds are formed between Met-8' to Phe-2' of the
20   peptide and Gln-104 to Lys-110 of PapD, thus extending the β-
sheet of PapD out into the peptide.

Apart form the C-terminal residues Phe-2' and Pro-1' there
are relatively few contacts between the side chains of the
peptide and PapD. The major interactions are provided by the
25   main chain hydrogen bonds to strand G1. There are, however, a
number of hydrophobic interactions within the β-sheet, in
particular between the peptide's Met-8' and Met-6' with Leu-
103, Ile-105 and Leu-107 of strand G1.

Calculations revealed that the four hydrophobic peptide side
30   chains of residues 2', 4', 6', and 8' contribute 20% of the
total buried surface area (582 Å^2) between the peptide and
the protein. Therefore, even though the major stabilization
of the complex is provided via hydrogen bonding, hydrophobic
interactions are not insignificant, and it is believed that
35   they provide part of the explanation for the specificity of

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PapD for pilus related peptides and subunits. Experimental support of this theory is provided by the reduced binding of *PapD* to the peptide G2'-21'amide as compared to the G1'-19'WT peptide (Fig. 1B and table C). Hydrogen bonding of the COOH-

5 terminus of the G2'-21'amide (which lacks Pro-1') to Arg-8 and Lys-112 of *PapD* allows main chain hydrogen bonding, but dislocates the four hydrophobic side chains in the peptide from their subsites in *PapD*, resulting in a reduction in binding strength.

10 Within the crystal the *PapD*-peptide β sheet was extended even further as a result of non-crystallographic twofold symmetry which placed a second *PapD*-peptide complex adjacent to the first so that the two bound peptide chains interacted as antiparallel β strands. In the present model, eight main

15 chain hydrogen bonds are formed between the two peptides; see the following table:

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Table B

PapD₁	Peptide₁	Peptide₂	PapD₂
		Pro-1'-COOH	Arg-8-NH₂
		Pro-1'-COOH	Lys-112-NZ
		Phe-2'-NH	Lys-110-C=O
	Glu-13'-NH	Ser-3'-C=O	
	Glu-13'-C=O	Ser-3'-NH	
		Leu-4'-C=O	Lys-110-NH
		Leu-4'-NH	Gln-108-C=O
	Ser-11'-NH	Val-5'-C=O	
	Ser-11'-C=O	Val-5'-NH	
		Met-6'-C=O	Gln-108-NH
		Met-6'-NH	Ala-106-C=O
	Ser-9'-NH ... ?	Thr-7'-C=O	
	Ser-9'-C=O ... ?	Thr-7'-NH	
Gln-104-C=O	Met-8'-NH		
		Met-8'-C=O	Ala-106-NH
Ala-106-NH	Met-8'-C=O		
		Met-8'-NH	Gln-104-C=O
	Thr-7'-NH ... ?	Ser-9'-C=O	
	Thr-7'-C=O ... ?	Ser-9'-NH	
Ala-106-C=O	Met-6'-NH		
Gln-108-NH	Met-6'-C=O		
	Val-5'-NH	Ser-11'-C=O	
	Val-5'-C=O	Ser-11'-NH	
Gln-108-C=O	Leu-4'-NH		
Lys-110-NH	Leu-4'-C=O		
	Ser-3'-NH	Glu-13'-C=O	
	Ser-3'-C=O	Glu-13'-NH	
Lys-110-C=O	Phe-2'-NH		
Lys-112-NZ	Pro-1'-COOH		
Arg-8-NH₂	Pro-1'-COOH		

A mixed β sheet is thus created between the two complexes and extends over ten β strands (Fig. 5). No contacts are observed between the two non-crystallographically related PapD molecules themselves, both of which were positioned in similar environments within the crystal and possessed a similar

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number of intermolecular contacts. The calculated buried
surface area between the two non-crystallographically related
peptides was 520 Å^2, a value similar to the surface area
buried between the protein and peptide. This "dimerization"
5   appears to be a consequence of crystal packing, because all
evidence shows that PapD forms monomeric complexes with
peptides or with intact PapG in solution.

The hydrogen bonding pattern between PapD and the peptide
breaks at Ser-9', but the peptide remains in van der Waals
10   contact with PapD until Ser-11' where the peptide runs beyond
the F1-G1 loop, but remains hydrogen bonded to the non-
crystallographically related peptide as far as Glu-13' (table
B). The last resolved amino acid of the peptide is Gly-14'
which is positioned close to the binding cleft of the non-
15   crystallographically related PapD. the first five amino
terminal amino acids, including three positively charged
residues, had no density and therefore must have been dis-
ordered in the crystal structure. In agreement with this, the
peptide's amino terminus is not important for binding to PapD
20   in solution, since the G1'-7'WT peptide lacking the amino
terminal 12 amino acids is found to be an effective inhibitor
of PapD binding to the immobilized G1'-19'WT peptide (table C
and example 2).

The structures of the individual PapD domains in the peptide
25   complex are essentially the same as those of native PapD
(Holmgren and Brándén, 1989). However, there is a significant
movement of the domains with respect to each other with a 13°
jaw-closing or hinge bending motion making the angle of the
PapD boomerang more acute (Fig. 6). Whether or not this
30   conformational change is the result of binding of peptide or
different crystal packing between the two crystal structures
is unclear.

In the native PapD structure the electron density obtained
for the long F1-G1 loop is poor between residues 96 and 102,
35   suggesting that it is rather flexible and disordered in the

crystal. In the peptide complex, however, this loop is better resolved, indication that binding of the peptide makes this loop more rigid. Superimposing the NH_2-terminal domains of native PapD and the peptide complex shows that there is also

5   a significant difference in the F1-G1 loop position between the two structures (rms for the 110 NH_2-terminal $C\alpha$ atoms is 1.84 Å, with a maximum main chain movement of about 9 Å for Leu-103). In the peptide complex the loop is seen to twist at one end away form the β barrel of the NH_2-terminal domain

10   thus facilitating a more extensive contact between strand G1 and the peptide. As with the hinge-bending of the two domains, it is not yet possible to say with certainty whether this loop shift is a consequence of peptide binding or of crystal packing; the rather open conformation of the F1-G1

15   loop suggests that it may be largely the latter. Nevertheless, evidence that similar interaction between PapD and peptides or pilus subunits occur in solution is provided by protease protection experiments after the F1-G1 loop of PapD is protected from tryptic cleavage by the binding of both

20   native PapG and the G1'-19'WT peptide (example 2 and Fig. 2A).

EXAMPLE 2
Binding between PapD and the carboxyl terminus of other Pap peptides

25   G1'-19'WT, E1'-19'WT, F1'-19'WT, K1'-19'WT and H1'-19'WT peptides (see table 1) were synthesized corresponding to the 19 carboxyl terminal residues of P pilus subunit proteins PapG, PapE, PapF, PapK and PapH, respectively (see: Grant et al., 1992, and references cited therein).

Table 1

Name	SEQ ID NO:	Peptide sequence
G1'-19'WT	6	NH_2-GKRKPGELSGSMTMVLSFP-COOH
G1'-16'WT	7	NH_2-KPGELSGSMTMVLSFP-COOH
G1'-11'WT*	8	NH_2-SGSMTMVLSFP-COOH
G1'-7'WT	9	NH_2-TMVLSFP-COOH
G1'-19'SV	10	NH_2-GKRKP<u>V</u>ELSGSMTMVLS<u>S</u>P-COOH
G1'-19'amide	11	NH_2-GKRKPGELSGSMTMVLSFP-<u>CONH</u>$_2$
G2'-21'amide	12	NH_2-<u>EE</u>GKRKPGELSGSMTMVLSF-<u>CONH</u>$_2$
E1'-19'WT*	13	NH_2-QNLIAGPFSATATLVASYS-COOH
H1'-19'WT	14	NH_2-KKLEAGNYFAVLGFRVDYE-COOH
K1'-19'WT	15	NH_2-KSVVPGDYEATATFELTYR-COOH
F1'-19'WT	16	NH_2-GILNGGDFQTTASMAMIYN-COOH
MS	17	NH_2-YALAPNAVIPTSLALL-COOH

* Water insoluble peptides

The residues in the peptides were numbered starting with the carboxyl terminal residue as 1, and ending with the amino terminal residue. Peptides were also synthesized which deviated in length (G1'-16'WT, G1'-11'WT, G1'-7'WT) or sequence (G2'-21'amide, G1'-19'SV, G1'-19'NH$_2$) from the wild type PapG carboxyl terminal sequence. Using an enzyme-linked immunosorbent assay (ELISA), we tested the ability of PapD to bind to each peptide coated on wells of microtiter plates:

5 mg/ml stock solutions of peptides in water or 50% acetic acid were diluted to a concentration of 2.5 pmol/50 µl in PBS. 50 µl of the peptide solution was coated overnight onto microtiter wells (Nunc-Immuno Plate Maxisorp) at 4°C. The solutions in the plates were discarded, and the wells were blocked with 200 µl 3% bovine serum albumin (BSA, Sigma) in PBS (120 mM NaCl/2.7 mM KCl/10 mM phosphate buffer salts, pH 7.4) for 2 hours at 25°C. The plates were washed vigorously 3

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times with PBS and incubated with 50 μl of the indicated
amount of purified PapD (Lindberg et al, 1989). After 3
washes with PBS, the wells were incubated with a 1:500 dilu-
tion of anti-PapD rabbit antiserum (Lindberg et al, 1989) in
5   3% BSA/PBS for 45 min. at 25°C. After 3 washes with PBS, the
wells were incubated with a 1:1000 dilution of goat anti-
rabbit IgG coupled to alkaline phosphatase (Sigma) in 3%
BSA/PBS for 45 min. at 25°C. Following 3 washes with PBS and
3 washes with developing buffer (10 mM diethanolamine pH 9.5,
10   0.5 mM MgCl$_2$), 50 μl filtered p-nitrophenyl phosphate
substrate (Sigma) in developing buffer (10 mg/ml) was added.
The absorbance at 405 nm was read after 60 min of incubation
in the dark at 25°C.

In addition, the ability of water-soluble peptides to inhibit
15   PapD binding to G1'-19'WT-coated wells in a soluble inhibi-
tion ELISA were tested, since peptide conformations may have
been affected by binding to the plastic microtiter plates:

Microtiter wells were coated overnight at 4°C with 50 μl of
2.5 pmol/50 μl of the G1'-19'WT peptide. The wells were
20   washed with PBS and blocked with 3% bovine serum albumin
(BSA). A 25-fold molar excess of each test peptide was pre-
incubated with 100 pmoles PapD for 30 min. and the PapD-
peptide solution was then added to the coated wells and
incubated at 25°C for 45 min in the presence of 3% BSA/PBS.
25   The subsequent primary antibody, secondary antibody and
developing steps are described above. The ability of the
peptides to inhibit binding of PapD to the G1'-19'WT peptide
was calculated by dividing the amount of PapD binding in the
presence of peptide with the amount of PapD binding in the
30   presence of water. 0% inhibition includes values where bin-
ding was greater than that of PapD pre-incubated with H$_2$O.

The results of PapD binding to wild type, variant and differ-
ent length peptides are shown in Figs. 1A, B and C.

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As can be seen the peptides bound well to the PapG peptide,
moderately to PapE, PapF and PapK, whereas there was no
binding to PapH and the random hydrophobic peptide, MS. (Fig.
1A). These results suggest that the chaperone recognizes
5   PapG, PapE, PapF and PapK in part by binding to the carboxyl
terminus of these subunits. The inability of PapD to interact
with the PapH peptide indicates that PapD binds differently
to PapH, possibly due to PapH's function as a polymerization
terminator (Baga et al., 1987). The ability of a peptide to
10   inhibit PapD binding to immobilized G1'-19'WT generally
corresponded to the affinity that PapD had for the respective
immobilized peptide (Fig. 1, % inhibition), arguing that the
interaction of PapD with soluble and immobilized peptides is
similar.

15   As can be seen from example 1, the molecular basis of the
PapD-peptide interactions have been studied by co-crystalli-
zation of PapD with the G1'-19'WT peptide. In summary, the 19
amino acid peptide is anchored in the chaperone cleft by
hydrogen bonds between the peptide's carboxy terminus and
20   Arg-8 and Lys-112 which are invariant in all periplasmic
chaperones (Holmgren et al., 1992). The peptide bound to the
G1 strand of PapD as a parallel β-strand, forming at least 10
backbone hydrogen bonds, and resulted in an ordering of the
F1 to G1 loop.

25   Replacement of the carboxyl terminal proline on the PapG
peptide with an amide (G2'-21'amide) abolished binding to
PapD in solution and reduced binding of PapD to the immobi-
lized peptide by approximately 75% (Fig. 1B). In contrast,
substituting only the carboxylate group with an amide to
30   create the G1'-19'NH$_2$ peptide did not affect binding to PapD
in either the immobilized or soluble inhibition ELISA assays
(Fig. 1B). These results indicate that the terminal proline
probably is required to position the carboxylate group so
that it can form hydrogen bonds with the invariant Arg-8 and
35   Lys-112 cleft residues.

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PapD also binds to the immobilized shorter peptides G1'-16'WT and G1'-11'WT and the G1'-16'WT and G1'-7'WT peptides inhibited binding of PapD to the immobilized G1'-19'WT peptide in solution. Table C shows the ability of a 25-fold molar excess of the water soluble peptides to inhibit binding of 100 pmol/well of PapD to G1'-19'WT-coated wells. The % inhibition represents the percentage of PapD binding in the presence of the peptide compared to in the presence of water and are the average of two experiments performed in duplicate:

Table C

Peptide	% Inhibition
G1'-19'	63
G1'-7'	49
G1'-4'	0
MS	1
G2'-21'amide	0
G1'-19'amide	56

PapD does not bind to immobilized G1'-7'WT peptide, probably because this peptide is to short to bind to the microtiter well as well as PapD. It, thus seems that as few as 7 carboxyl terminal residues are necessary for PapD binding of a peptide. Together these results support a model where, in addition to the anchoring interaction of the carboxylate group in the PapD cleft, seven carboxyl terminal residues are required for "zippering" the peptide along the G1 strand of PapD during PapD-peptide complex formation.

Conserved residues phenylalanine and glycine residues at positions 2 and 14 from the carboxyl terminus were substituted by serine and valine, respectively, to create the

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peptide G1'-19'SV and decreased binding to PapD by 36%. This could result form less efficient coating or presentation in the microtiter plate since G1'-19'SV was as efficient a soluble PapD inhibitor as the peptide G1'-19'WT (Fig. 1B). Since these residues are critical for incorporation of PapG into the pilus, it is believed that they be more important for intersubunit polymerization interactions and pilus assembly than in PapD subunit interactions *in vivo*.

Partial digestion with trypsin cleave PapD in the F1-G1 loop at residues Leu-103 and Lys-99, respectively:

400 μg of PapD was partially digested by incubation with either 4,5μg trypsin or 0.45 μg chymotrypsin on PBS for 20 min. at 37°C. The PapD digests were applied to a C-18 HPLC column (Beckman) and 2 major fragments were eluted with a 0-100% acetonitrile gradient in 0.01% trifluoroacetic acid. The PapD fragments were identified by their molecular weight on SDS-PAGE and amino-terminal sequencing. The N-terminal amino acid sequences of the approximately 14 kDa tryptic and chymotryptic fragments were identified as residues 100-108 and 104-109 of PapD, respectively, corresponding to cleavage after Lys-99 (for trypsin) and Leu-103 (for chymotrypsin). The N-terminal sequences of the 11 and 12 kDa bands in the digests were identical to the N-terminal sequence of PapD.

The G1'-19'WT, G1'-16'WT and K1'-19'WT peptides, but not the G2'-21'amide peptide, reduced the rate of tryptic cleavage of PapD over time (Fig. 2A). These data argue that binding of PapD to the peptide altered contacting the loop and thereby protectin to form cleavage. This effect may be related to the ordering of the F1-G1 loop of PapD observed in the PapD-peptide crystal structure, and suggests that, in solution, both peptides extends along the G1 β-strand and interact with the F1-G1 loop of PapD.

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As described in Kuehn et al., Proc. Natl. Acad. Sci. USA 88,
10586 (1991), native PapD is able to bind to reduced, dena-
tured PapG and restore the PapD-PapG complex in vitro.
This reconstitution assay was used to determine the ability
5   of the peptides to inhibit PapD activity in vitro. The
limited solubility of the G1'-7'WT peptide unfortunately has
prevented the testing of this peptide in the assay. Increa-
sing amounts of the G1'-19'WT, G1'-16'WT and K1'-19'WT pepti-
des inhibit restoration of the PapD-PapG complex by PapD,
10 while the G2'-21'amide peptide has no effect (Fig. 2B). The
ability of the G1'-19'WT, G1'-16'WT and K1'-19'WT peptides to
prevent PapD form binding to PapG indicated that these pepti-
des bound in the subunit binding site of PapD.

The ability of peptides to inhibit restoration of the PapD-
15 PapG complex by PapD was tested as follows.: 0.3 μg of PapD-
PapG complex was reduced and denatured by incubation at 25°C
for 20 min with 4 M urea/10 mM dithiothreitol (DTT), 1.2 μg
(50 pmol) of PapD was incubated at 25°C for 10 min with 5-
14.5 μg (2.5-7.25 nmol) of peptide. The PapD-peptide solution
20 was then added to the reduced, denatured PapD-PapG, incubated
at 25°C for 10 min, and applied to an IEF 3-9 gel (Pharma-
cia). The amount of PapD-PapG restored in each sample was
quantitated by densitometry of the silver stained IEF band
corresponding to the pI of the DG complex.

25 Site directed mutations in strictly conserved cleft residues
of PapD predicted to be critical in the PapD-peptide interac-
tion were constructed to test whether the PapD-peptide crys-
tal structure is a reflection of part of the PapD-pilus
subunit interaction interface (the positions of the mutations
30 are indicated in Fig. 3). Highly conserved Thr-7 was changed
to a valine (Thr-7-Val) in order to test whether its hydroxyl
group formed hydrogen bonds critical for PapD binding to
subunits.

This mutation removed the hydroxyl group while maintaining
35 the steric volume of the side chain. Mutations in Lys-112 and

Arg-8 were designed to test whether hydrogen bonding to the
terminal carboxylate group of pilus subunits is a critical
feature of the chaperone recognition process. The invariant
Lys-112 residue was changed to an alanine (Lys-112-Ala) to

5 remove the charged side chain and to a methionine (Lys-112-
Met) to replace the charged group with a hydrophillic group
while maintaining the side chain packing. The invariant Arg-8
has been shown previously to be required for the ability of
PapD to bind subunits and mediate pilus assembly in vivo

10 (Slonim et al, 1992). Glu-167 /E167, a variable residue in
domain 2 of PapD (Fig. 3), does not appear to be involved in
the interaction between PapD and the peptide in the crystal
structure and mutations in this residue have been shown to
have little or no effect on PapD function in vivo (Slonim et

15 al, 1992). E167 was changed to histidine (E167H) to test
whether this negatively charged residue at the lip of domain
2 has any role in the PapD-peptide interaction. All of the
mutants were secreted into the periplasmic space as stable
proteins similar to wild type PapD. In addition, the elution

20 profile form a cation-exchange FPLC column and the electro-
phoretic properties of the purified mutant PapDs were similar
to the wild type protein, supporting the prediction that
these mutations would not affect the overall structure of
PapD (data not shown).

25 The ability of the mutant chaperones to bind pilus subunits
and modulate pilus assembly in vivo was correlated to their
ability to bind to the G1'-19'WT peptide and to PapG in
vitro. Wild type PapD bound the G1'-19'WT peptide to cause a
mobility shift towards the negative electrode in a native

30 polyacrylamide gel assay (Lam and Calderwood, 1992), probably
due to a net positive charge increase in the PapD-peptide
complex. In contrast, when Arg-8-Gly, Arg-8-Ala and Lys-112-
Ala PapD mutants were incubated with the G1'-19'WT peptide
they did not cause a mobility shift in the native-PAGE assay,

35 indicating that these mutations in PapD abolished peptide
binding. Similarly, mutations in Arg-8 and Lys-112 abolished
the ability of PapD to bind to PapG and reconstitute the

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PapD-PapG complex in vitro (Fig. 7) and to bind to pilus subunits and mediate pilus assembly in vivo (cf. table 2).

Table 2

Residue class	Mutation	HA titer*	Pilus assembly+	Subunit stabilization#				
				PapA	PapE	PapG	PapF	PapK
Invariant	Arg-8-Gly	None	-	-	-	-	-	-
	Arg-8-Ala	None	-	+	-	+	-	+
	Arg-8-Met	None	-	+ +	-	+ +	-	+ +
	Lys-112-Ala	None	-	+	-	+	-	+
	Lys-112-Met	None	-	+ +	-	-	-	-
Conserved	Thr-7-Val	64	+ + +	+ - + +	+ + + +	- + + +	+ + + +	+ + + +
Variable	Glu-167-X§	128	+ + + +	+ - + +	ND	ND	+ + + +	+ + + +

*   The hemagglutionation (HA) titer was quantitated after induction with 0.01 mM IPTG. The HA titer represents the greatest bacterial dilution which still agglutinates erythrocytes. HB101/pPAP37 expressing wild type PapD has an HA titer of 128.

+   The amount of pili assembled were released from the cells and quantitated after induction with 0.01 mM IPTG. "-" indicates no pili were detected; the degree of piliation of HB101/pPAP37 expressing wild type PapD corresponds to "+ + + +".

Chaperone-assisted periplasmic stabilization of pilus subunits PapA, PapE, PapF, PapK and PapG was determined as described previously (Slonim et al., 1992); "-" indicates no stabilization of the subunit; the number of +'s indicate the degree of subunit stabilization as compared to wild type PapD. ND indicates not determined.

§   X indicates mutations to His, Asp, Thr, or Gly.

The remarkable consistency of the in vitro and in vivo results and the crystal structure argue that anchoring of pilus subunits by conserved residues Arg-8 and Lys-112 is a critical part of the interaction between PapD-like chaperones and pilus biogenesis.

Site-directed mutagenesis was performed using the Bio-Rad in vitro mutagenesis kit following the manufacturers instructions. The following primers (non-coding strand) were used to introduce mutations in the papD gene (SEQ ID NO: 1):

Thr-7-Val   5'-GTCAAACACCGCCGGAACTCGTCCAGGCGA-3' SEQ ID NO: 3

Lys-112-Ala 5'-CGGGCGATAAAAAGAGCTATTTGGTCTG-3' SEQ ID NO: 4

Lys-112-Met 5'-GCGATAAAAAGCATTATTTCCTCTG-3'   SEQ ID NO: 5

Mutations in papD were confirmed by sequencing and the
altered papD genes were cloned into vector pMMB91 under the
5   inducible P$_{tac}$ promotor as previously described for other
papD mutations (Slonim et al, 1992). The plasmids were named
pThr-7-Val, pLys-112-Ala and pLys-112-Met according to the
residue number and mutated amino acid. Plasmid pLS101 is an
isogenic construct containing the wild type papD gene;
10   plasmid pE167H, pR8G and pR8A contain genes encoding PapD
with point mutations changing Glu-167 to His and Arg-8 to Gly
and Ala, respectively.

The data presented in this example and example 1 demonstrate
that the PapD-peptide interactions found in the crystal
15   structure reflects the PapD-pilus subunit interaction inter-
face in vivo. These interactions define for the first time a
mechanism whereby chaperones utilize their immunoglobulin-
like domains in a novel recognition paradigm to bind to a
diverse group of proteins. PapD residues Arg-8 and Lys-112,
20   located in β-strands in the cleft between the two domains,
play crucial roles in chaperone recognition by servings as
molecular anchors for the carboxyl terminus of pilus pro-
teins. The fact that Arg-8 and Lys-112 are highly conserved
amongst periplasmic chaperones indicates that they probably
25   have a universal role in forming the "active site" that binds
to the terminal carboxylate group of other protein subunits.
Backbone hydrogen bonds along the β-strand of PapD
subsequently provide strong, sequence-independent interac-
tions along the length of the carboxyl terminus of the sub-
30   unit. The "zippering" interaction of the conserved alter-
nating hydrophobic residues of the carboxyl terminus of the
pilus subunit with the conserved alternating hydrophobic
residues in the G1 β-strand of the chaperone adds strength
and specificity to the binding. Therefore, unlike other
35   immunoglobulin-like proteins (Amit et al, 1988 and de Vos et
al, 1992), PapD utilizes the β-strand and interdomain cleft

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features of its immunoglobulin-like structure to provide a
recognition mechanism for binding to several different pro-
teins. Variable residues in loop regions of antibodies pro-
vide the exquisite specificity necessary from an extensive
5   antibody binding repertoire. Similarly, residues in the F1-G1
loop of PapD may provide specificity to chaperone binding
since these have been found to vary in length and composition
amongst members of the periplasmic chaperone family (Holmgren
et al, 1992).

10   EXAMPLE 3
       *Designing of compounds capable of binding to the binding site
       of PapD*

       Having determined the location of a promising binding site
       for inhibitory ligands as described in Examples 1 and 2, the
15   computer programs 'PLIM' and 'PLIM_DBS' (developed by Symbi-
       com AB) were used to find templates for families of compounds
       capable of binding to the binding site (see the general
       description above).

       PLIM was run on a 20 Å box around the Arg-8 region of the
20   high-resolution structure of PapD, searching for low-energy
       binding positions for NH, NH_2, O, OH and C probes. The PLIM
       runs resulted in a number of suggested positions and orienta-
       tions of favourable chemical groups (site points) in the
       region near Arg-8 and Lys-112.

25   Approximately 50 sites were identified, and then triplets and
       quartets of these site points were selected for their mutual
       compatibility, that is to say they must have appropriate
       geometric relationships - distances and orientations that
       could be found in a real molecule.

30   A search for potential ligands was then made by searching a
       database for known molecular structures that match the posi-
       tions of these groups of site points, using PLIM_DBS.

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The best of these database hits were examined visually using a computer-graphic modelling system, and the most promising of these were selected according to a wealth of physico-chemical reasoning.

5   Modifications were made as described above to improve binding and simplify synthesis. For example, phenyl rings were added to give better complementarity to the hydrophobic surface of the binding site, charged groups were added to bind to charged groups on the protein that had been outside the scope
10 of Plim, frequently toxic sub-structures were removed, and groups were replaced with functionally similar ones for synthetic reasons. Many such judgements were made, resulting in two families of compounds (hdo and bpy) with differing combinations of characteristics.

15 The efficacy of these modifications was finally assessed using molecular-dynamics free energy calculations as described herein to study the stability of the protein-ligand complex (Åqvist et al., 1994).

Two structures from one family and one from the second family
20 were then deemed worthy of synthesis and testing.

The development of a number of the member of these families of compounds will now be described in detail.

The first family (herein referred to as hdo) was derived from a database entry for 6-hydroxydopamine, denoted hdo_0 (Fig.
25 8). The molecule binds to the PapD side chains of Arg-8 and Lys-112, and to the backbone of Lys-110 through hydrogen-bond donation from the hydroxyl groups. The primary-amino group hydrogen-bonds to the side chain of Thr-170.

Derivative hdo_1 (Fig. 8) was created from this base struc-
30 ture by replacing an hydroxyl group with a carboxylic acid that can have a charge interaction with Arg-8 and Lys-112, and replacing the hydroxyl para to this position with a new

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phenyl ring, in order to fill out the hydrophobic cleft in
the protein formed by the residues Ile-111, Leu-4 and part of
Thr-7. hdo_4 (Fig. 8) was created from hdo_1 by replacing the
original aromatic ring with a sugar, attaching the hydroxyl,
5   primary amine and carboxylic acid substituents in such a way
as to maintain similar relative geometries, and then adding
hydroxyl groups to one side of the new benzene ring so as to
present a hydrophillic face to the solvent in this region.

At this point the structure looked convincing enough to
10   invest time in a molecular-dynamics simulation, the results
of which suggested that the amino group would not in fact
bind to the gamma-carbon of Thr-7 of PapD, but would prefe-
rentially point into solution, satisfying its hydrogen-bon-
ding requirements with water molecules rather than with the
15   protein side-chain. Thus, the amine group was replaced by the
singly donating hydroxyl group, a step that also simplified
synthesis, the result being structure hdo_6 (Fig. 8).

Due to the perceived probability of toxicity due to the
hydroxyl substitution pattern on the benzene ring, the pa-
20   ra-hydroxyl group was removed to give structure hdo_7 (Fig.
8), and the remaining hydroxyl group on this ring was
replaced by a nitro group for further ease of synthesis
(hdo_8, Fig. 8).

A final compound with the previous nitro group left off was
25   also considered a possibility, and was known as hdo_9 (Fig.
8).

Another molecular-dynamics simulation was run, on hdo_9, the
results of which suggested that now the predicted interac-
tions would indeed be made and maintained in solution. A
30   statistical analysis of the trajectories with the program
"Miss_Fit" showed that the conformation of the ligand did not
change substantially during the run, and changes that did
occur were compensated for by complementary shifts in the

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protein structure that maintained all significant interactions.

Dynamics simulation of hdo_8 was complicated by the lack of availability of suitable molecular-mechanics parameters for
5   the meta-nitro substituted ring, which made assessment through molecular-dynamics simulations harder to rely upon, and this influenced the decision to pursue hdo_9.

The range of possibilities that were considered during the design phase of the hdo family are summarised in Fig. 9.

10   The second family, known as bpy, was derived from a database entry for (methyl-O,N,N-azoxy)-methyl-β-D-glucopyranoside cycasin (Kawaminami et al, 1981) denoted as bpy_0 herein:
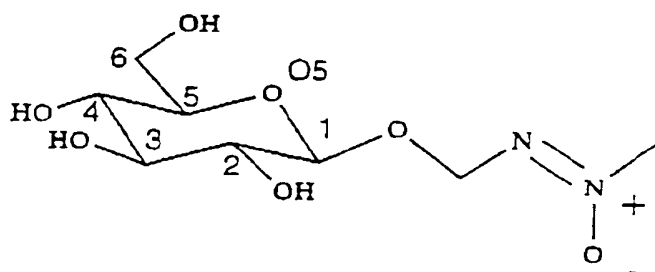
bpy_0

The conformation stored in the database was non-optimal for
15   binding to the *PapD* site, though it was close enough to be picked out by the Plim_DBS search. Small adjustments made to the internal torsion angles improved the binding pattern somewhat.

It is interesting to note that there are precedents for the
20   approach of using a central carbohydrate as a scaffold to which functionalities are attached as to mimic a peptide (Hirschmann et al, 1992).

Derivative bpy_1 was then created by adding a phenyl ring to the 6-position, in order to fill out the hydrophobic cleft in

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the protein. The 4-OH group should form a favourable dipole
interaction with the electron-rich centre of the 6-phenyl
group, and together with the 6-OH...C5 intramolecular hydrogen
bond should stabilize the desired C5-C6 rotamer.

5   It was decided that the Lys-112 and Arg-8 side chains that
should interact with the hydroxyl group at the 2-position in
the ligand do so even more with a charged group, and there-
fore bpy_2 was created by replacing this hydroxyl with a
sulphate, bpy_3 by replacing it with a cyclic phosphate, and
10   bpy_5 by replacing it with a non-cyclic phosphate.

bpy_4 was created by removing the 3-hydroxyl group from BPY_2
so that the sulphate group had a better chance of interacting
with the Arg-8 side chain, and bpy_6 was analogously created
by removing the 3-hydroxyl group from bpy_5. The following
15   formula shows bpy_1-bpy_6:
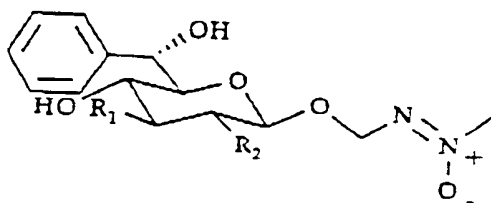
R_1	R_2	
-OH	-OH	bpy_1
-OH	$-OSO_2^-$	bpy_2
$-O-PO_2^--O-$		bpy_3
-H	$-OSO_2^-$	bpy_4
-OH	$-OPO_2^{2-}$	bpy_5
-H	$-OPO_2^{2-}$	bpy_6

Consideration was then given to a small hydrophobic pocket
and patches or hydrophobic residues exposed to the solvent
near position 1 on the ligand. An aglycon that could make

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contact with these parts of the protein and preferably also
interact with solvent was sought. These considerations led to
the replacement of the azoxy group at position 1 with a 2-
hydroxy-4-methylcyclopentyl group, resulting in bpy_7:
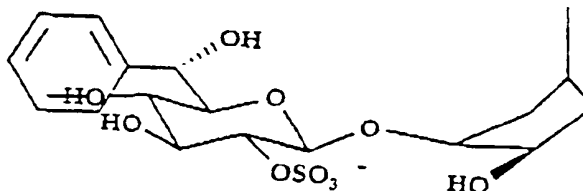
bpy_7

At this point, a molecular dynamics run was made, the results
of which suggested that the structure bound well, but that
the expected hydrophobic contacts between the cyclopentane
and the protein were not maintained.

10 Thus, bpy_9 was created from bpy_7 with the cyclopentane
group removed, replaced by an α-methyl group for internal
stability. Instead, further hydrophobic contact was attempted
by the addition of an ethoxy group axial on position 2. The
charge was moved from equatorial 2 to axial 3, a move

15 stabilised by the inclusion of a NH-link that can donate a
hydrogen bond to the oxygen at axial 1. The stereochemical
arrangement of functionalities on positions 1-3 now confers
conformational rigidity to the structure, as well as a sim-
plification of synthesis.

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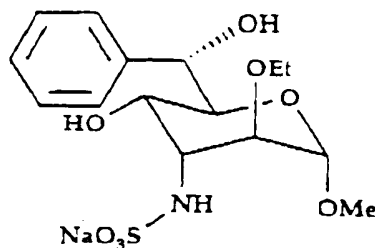
bpy_9

More dynamics were run, this time suggesting that bpy_9 would
form a stable complex with the protein, similar to the way
that was envisaged during the design.

5   *Further developments*

By using reasoning in line with the above-described, more
compounds have been designed (both from the hdo_family and
from the bpy_family). The compounds of interest are those
described by the general formulas I and II herein. The most
10 promising compounds resulting from the analyses have been
synthesized, cf. example 4.

In the further development of designed compounds using the
above-described methods it has interestingly been found that
the stability of the binding between the designed compounds
15 and *PapD* seems to be only limitedly dependent on the interac-
tion between the compound and the Arg-8 residue, even though
Arg-8 is essential for the *in vivo* function of *PapD*. On the
other hand, the stability of the interaction between the
designed compounds and *PapD* is still very much dependent on
20 the interaction with Lys-112 as well as with the β-strand.
These observations confirm the surprising observation that
the stable binding between *PapD* and pilus subunits is very
much dependent on the interaction between the β-sheets of the
two molecules.

EXAMPLE 4

Synthesis of compounds of the hdo family capable of binding to the binding site of PapD

General methods

5 ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ at 300 and 75 MHz respectively on a Varian Gemini 300 spectrometer unless otherwise stated. Signals from undeuterated solvent at 7.25 and 77.0 ppm respectively were used as internal reference signals. Optical rotations were measured using a Perkin Elmer
10 241 polarimeter.

Thin layer chromatography was performed on Merck *DC-Fertig-platten* (Kiselgel 60 F_{254} 0.25 mm) and spots visualized by spraying with 10% sulphuric acid followed by charring at elevated temperature and/or spraying with molybdatophosphoric
15 acid hydrate/Ce$(SO_4)_2$/dil. H_2SO_4 followed by heating (molybdatophosphoric acid hydrate = $H_3[P(Mo_3O_{10})_4]$ x H_2O, E.Merck, Darmstadt, Germany)

Ethyl 2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyranoside

20 Ethyl 2,3-O-dibenzoyl-4,6-O-benzylidene-1-thio-β-D-glucohexopyranoside (150 mg, 0.29 mmol) was dissolved together with borane-dimethylamine complex (68 mg, 1.16 mmol) in dry toluene (20 Å). Powdered 4 Å molecular sieves (180 mg) was added and the mixture was stirred at room temperature for 20
25 minutes. Aluminum trichloride (154 mg, 1.16 mmol) was added and after disappearance of the starting material (approx. 10 min., silica tlc toluene/acetonitrile 4:1) the mixture was filtered, treated with Dowex(H$^+$) ion-exchange resin until the solution was clear, and filtered again. The filtrate was
30 concentrated and co-evaporated twice with methanol to yield 200 mg residue which upon chromatography on silica (toluene-acetonitrile 8:1) gave 60 mg, 40% of ethyl 2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyranoside.

^{1}H-NMR (CDCl$_3$, δ): 8.0-7.2 (mm, 15H), 5.75 (t, 9.4 Hz, 1H, H3), 5.37 (t, 9.8 Hz, 1H, H2), 4.74 (d, 9.8 Hz, 1H, H1), 4.61 (s, 2H), 4.02-3.95 and 3.85-3.76 (2 bm, 2H, 2H6) 3.94 (t, 9.5 Hz, 1H, H4), 3.63 (ddd, 9.7 Hz, 3.9 Hz, 2.6 Hz, 1H, H5), 2.74 (2q, 7.5 Hz, 2H), 1.25 (t; 7.5 Hz, 3H).

Ethyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-gluco-hexopyranoside

Ethyl 2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyrano-side (200 mg, 0.38 mmol) was dissolved in an ice-cooled mixture of pyridine and acetic anhydride (1:1, 6 Å). The mixture was stirred and attained at room temperature over-night. The mixture was diluted with dichloromethane, washed with satd. aq. sodium hydrogen carbonate and water and dried over sodium sulphate. The solvents were removed under reduced pressure and the residue was subjected to flash chromatogra-phy on silica gel (toluene-acetonitrile 6:1) to yield 204 mg, 93% of ethyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyranoside.

^{1}H-NMR (CDCl$_3$, δ): 8.0-7.2 (mm, 15H), 5.75 (t, 9.2 Hz, 1H, H3), 5.40 (t, 9.8 Hz, 1H, H2), 4.71 (d, 10.0 Hz, 1H, H1) 4.59 and 4.55 (2d, 10.8 Hz, 2H, bzl-CH$_2$), 4.44 (dd, 12.0 Hz, 1.9 Hz, 1H, H6), 4.27 (dd, 12.2 Hz, 4.6 Hz, 1H, H6') 3.87 (t, 9.5 Hz, 1H, H4), 3.77 (ddd, 9.7 Hz, 4.6 Hz, 1.9 Hz, 1H, H5), 2.73 (m, 2H), 2.09 (s, 3H), 1.26 (t, 3H).

Methylglycolyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-β-D-glucohexopyranoside

6-O-Acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopy-ranoside (600 mg, 1.04 mmol) and N-iodosuccinimide (468 mg, 2.08 mmol, dried 3h, < 0.1mbar) were dissolved in dry acetonitrile (7 Å, passed through alumina) at room tempera-ture. Methyl glycolate (161 µl, 2.08 mmol) was added and the mixture was stirred for 25 min at room temperature and then cooled on an ice-bath. Trifluoromethylsulphonic acid (18 µl,

0.21 mmol) was carefully added. Complete conversion of starting material had occurred after 10 minutes (tlc: silica toluene-acetonitrile 4:1). The reaction was quenched by addition of triethylamine, the mixture was diluted with dichloromethane, washed with water, sodium hydrogen carbonate and dried over sodium sulphate. The solvents were removed under reduced pressure and the residue subjected to chromatography on silica (toluene-acetonitrile 11:1) to yield 560 mg, 86% of ethyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyranoside.

Ethyl 2,3-O-Dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyranoside (hdo_9:1)

Ethyl 2,3-O-dibenzoyl-4,6-O-benzylidene-1-thio-β-D-glucohexopyranoside (Chemical Abstracts, RN 149563-08-6 or Hällgren and Widmalm, 1993) was dissolved together with borane-dimethylamine complex (68 mg, 1.16 mmol) in dry toluene (20 ml) (reaction "a" in Fig. 6). Powdered 4 Å molecular sieves (180 mg) were added and the mixture was stirred at room temperature for 20 minutes. Aluminium trichloride (154 mg, 1.16 mmol) was added and after disappearance of the starting material (approx. 10 min., silica tlc toluene/acetonitrile 4:1) the mixture was filtered, treated with Dowex(H⁺) ion-exchange resin until the solution was clear, and filtered again. The filtrate was concentrated and co-evaporated twice with methanol to yield 200 mg residue which upon chromatography on silica (toluene-acetonitrile 8:1) gave 60 mg, 40% of hdo_9:1.

^{1}H-NMR (CDCl$_3$,δ): 8.0-7.2 (mm, 15H), 5.75 (t, 9.4Hz, 1H, H3), 5.37 (t, 9.8Hz, 1H, H2), 4.74 (d, 9.8Hz, 1H, H1), 4.61 (s, 2H), 4.02-3.95 and 3.85-3.76 (2 bm, 2H, 2H6) 3.94 (t, 9.5Hz,1H, H4), 3.63 (ddd, 9.7Hz, 3.9Hz, 2.6Hz, 1H, H5), 2.74 (2q, 7.5Hz, 2H), 1.25 (t; 7.5Hz, 3H).

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Ethyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-gluco-hexopyranoside (hdo_9:2)

Ethyl 2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyrano-
side (hdo_9:1) (200 mg, 0.38 mmol) was dissolved in an
5 ice-cooled mixture of pyridine and acetic anhydride (1:1, 6
ml). The mixture was stirred and attained room temperature
overnight. The mixture was diluted with dichloromethane and
washed with saturated aqueous sodium hydrogen carbonate and
water, dried over sodium sulphate. the solvents were removed
10 under reduced pressure and the residue was subjected to flash
chromatography on silica gel (toluene-acetonitrile 6:1) to
yield 204 mg, 93% of hdo_9:2.

^{1}H-NMR (CDCl$_3$, δ): 8.0-7.2 (mm, 15H), 5.75 (t, 9.2Hz, 1H,
H3), 5.40 (t, 9.8Hz, 1H, H2), 4.71 (d, 10.0Hz, 1H, H1) 4.59
15 and 4.55 (2d, 10.8Hz, 2H, bzl-CH2), 4.44 (dd, 12.0Hz, 1.9Hz,
1H, H6), 4.27 (dd, 12.2Hz, 4.6Hz, 1H, H6') 3.87 (t, 9.5Hz,
1H, H4), 3.77 (ddd, 9.7Hz, 4.6Hz, 1.9Hz, 1H, H5), 2.73 (m,
2H), 2.09 (s. 3H), 1.26 (t, 3H).

Methylglycolyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-β-D-glu-
20 *cohexopyranoside (hdo_9:3)*

6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopy-
ranoside (hdo_9:2) (600 mg, 1.04 mmol) and N-iodosuccinimide
(468 mg, 2.08 mmol, dried 3h, < 0.1 mbar) were dissolved in
dry acetonitrile (7 ml, passed through alumina) at room
25 temperature. Methyl glycolate (161 μl, 2.08 mmol) was added
and the mixture was stirred for 25 min at room temperature
and then cooled on an ice-bath. Trifluoromethylsulphonic acid
(18 μl, 0.21 mmol) was carefully added. Complete conversion
of starting material had occurred after 10 minutes (tlc:
30 silica toluene-acetonitrile 4:1). The reaction was quenched
by addition of triethylamine and the mixture was diluted with
dichloromethane, washed with water, sodium hydrogen carbonate
and dried over sodium sulphate. The solvents were removed
under reduced pressure and the residue subjected to chroma-

tography on silica (toluene-acetonitrile 11:1) to yield 560
mg, 86% of (hdo_9:3).

Sodium Glycolyl 4-O-benzyl-β-D-glucohexopyranoside (hdo_9)

Methylglycolyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-β-D-
5   glucohexopyranoside (hdo_9:3) (350 mg, 0.59 mmol) was dis-
solved in 7 Å 25 mM sodium methoxide (methanol) and stirred
at room temperature for 20 h or until only a very slow-moving
spot could be detected on tlc by sulphuric acid charring
(silica, toluene-acetonitrile 4:1). A small sample of this
10 solution (=i) was retained as tlc reference before the methyl
glycolate hydrolysis. Sodium hydroxide (15 mg) and water (200
μl) was added and stirring was continued for 8h or until all
"i" was consumed (tlc silica, ethyl acetate-ethanol-acetic
acid 8:3:1). The reaction was quenched by bringing the pH to
15 4.2 with acetic acid. The solvents were removed under reduced
pressure and the residue (650 mg) was subjected to flash
chromatography on silica (ethyl acetate-ethanol-acetic acid
8:3:1). The material in the R_f-range 0.15 - 0.25 was col-
lected and concentrated to yield 178 mg, 92% of amorphous
20 white solid.

^{1}H-NMR (CD$_3$OD, δ relative to methanol at 3.31 ppm): 7.45 (m,
5H), 4.65 (d, 11.0 Hz, 1H), 4.35 (d, 16.0 Hz, 1H), 4.32 (d,
7.9 Hz, 1H, H1), 4.11 (d, 15.8 Hz, 1H), 3.82 (dd, 2.0 Hz,
12.1 Hz, 1H, H6), 3.65 (dd, 4.8 Hz, 12.1 Hz, 1H, H6), 3.59
25 (t, 9.0 Hz, 1H, H3 or H4), 3.40 (t, 9.1 Hz, 1H, H3 or H4)

This material still contained much acetic acid, so 50 mg was
redissolved in water and freeze-dried to give 30 mg of sub-
stance free acetic acid but with a water content (^{1}H-NMR) of
24.5 mol. eq.

30 This material was again redissolved in water and 1 equivalent
of sodium hydroxide was added, the solution was freeze-dried
to give 22 mg of the title compound.

^{1}H-NMR (CD$_3$OD, δ relative to methanol at 3.31 ppm): 7.40 -
7.22 (m, 5H), 4.95 (d, 10.8 Hz, overlap by residual HDO at
4.93), 4.65 (d, 11.0 Hz, 1H), 4.34 (d, 16.0 Hz, 1H), 4.32 (d,
7.5 Hz, 1H, H1), 4.10 (d, 15.8 Hz), 3.82 (dd, 1.8 Hz, 11.9
5   Hz, 1H, H6), 3.66 (dd, 4.8 Hz, 12.1 Hz, 1H, H6), 3.59 (t, 8.8
Hz, 1H, H3), 3.41 (t, 9.7 Hz, 1H, H4), 3.15-3.06 (m, 2H, H5,
H2).

^{13}C-NMR (CD$_3$OD, δ relative to methanol at 49.0 ppm): 176.1,
140.0, 129.3, 129.1, 128.6, 104.3, 79.1, 78.1, 77.2, 75.7,
10 75.2, 68.4, 62.3.

2-(Hydroxy)ethyl 4-O-benzyl-β-D-glucopyranoside (hdo_23)

A mixture of N-Iodosuccinimide (0.109 g, 0.484 mmol) and
triflic acid (6.6 μl, 0.0744 mmol) in dichloroethane-diethyl
ether (2 ml, 1:1) was added to a stirred mixture of the
15 thioglycoside ethyl 6-O-acetyl-2,3-O-benzoyl-4-O-benzyl-1-
thio-β-D-glucopyranoside (hdo_9:2) (0.210 g, 0.372 mmol),
ethylen glycol (0.125 ml, 2.232 mmol), and molecular sieves
(0.3 g, 4 Å) in dichloromethane-diethyl ether (3 ml, 1:2) at
room temperature during 5 min. After 25 min TLC showed
20 approx. 20 % conversion of the thioglycoside, thus more
N-Iodosuccinimide (0.109 g, 0.484 mmol) and triflic acid (6.6
μl, 0.0744 mmol) in dichloroethane-diethyl ether (2 ml, 1:1)
was added during 5 min. After additional 30 min. the reaction
mixture was filtered through a layer of Celite into an aque-
25 ous solution of sodium hydrogen carbonate and sodium
bisulphite. The organic layer was separated, washed with
water, dried (Na$_2$SO$_4$) and concentrated. The residue was
coevaporeted twice with toluene, dissolved in methanol con-
taining sodium methoxide (10 ml, 0.2 M), kept 1 h at 50°C,
30 and evaporated. Column chromatography (SiO$_2$, chloroform-me-
thanol-water, 100:15:1) of the residue gave hdo_23 (33 mg, 30
%).

$[\alpha]_D^{22}$ + 0.9° (c 1.4, methanol)
TLC: Rf 0.29 (chloroform-methanol-water, 100:15:1).

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^{13}C-NMR (CD$_3$OD, δ: relative to methanol at 49.0 ppm) δ:
139.9, 129.2, 129.0, 128.6, 104,4 (C-1), 79.2, 78.2, 77.0,
75.7, 75.4, 72.3, 62.3, 62.3 ppm.

^{1}H-NMR (CD$_3$OD, δ: relative to methanol at 3.31 ppm)) δ:
5   7.45-7.25 (m, 5H), 4.95 (d, 11.0 Hz, 1H, benzylic),4.65 (d,
11.0 Hz, 1H, benzylic), 4.29 (d, 7.7, Hz, 1 H, H-1) ppm.

EXAMPLE 5

*Synthesis of compounds of the bpy family capable of binding
to the binding site of PapD*

10   *Methyl 4'-methoxyphenylmethylene-α-D-mannohexopyranosides,
mixture of 4,6-, 2,3-endo and 2,3-exo monoacetals*

(See: Patroni et al, 1988).
Methyl α-D-mannohexopyranoside (38.8 g, 200 mmol) and p-
toluenesulphonic acid (200 mg) were dissolved in dimethylfor-
15   mamide (300 ml) and stirred at 100°C under a nitrogen stream.
4-methoxy-benzaldehyde dimethyl acetal (37.7 g, 240 mmol) in
dimethylformamide (300 ml) was added dropwise under 3h. Since
starting material still remained (tlc, silica, ethyl ace-
tate), stirring and heating was continued for 2h. The compo-
20   sition of the reaction mixture did not show any visible
change at that time (tlc, silica, ethyl acetate) and the
reaction was quenched with potassium carbonate (2.15 g). The
solvent was removed under reduced pressure and the residue
was filtered through silica (ethyl acetate) to remove salts
25   and starting material. The concentrated eluents deposited
monoacetals (^{1}H-NMR) on trituration with methyl t-butyl ether
(11 g, 18%), which was collected by filtration. The filtrates
were subjected to flash chromatography to yield additional 20
g of the monoacetal mixture (yield 51%) and 17 g of the
30   2,3;4,6-di acetals (endo/exo = 1:1).

Methyl 2-O-ethyl-4,6-O-(4'-methoxy)phenylmethylene-α-D-manno-hexopyranoside (bpy_9:1)

Methyl 4'-methoxyphenylmethylene-α-D-mannohexopyranosides, mixture of 4,6-, 2,3-endo and 2,3-exo monophenylmethylene
5   acetals (11.3 g, 36.2 mmol), ethyl iodide (4.35 ml, 54.3 mmol) and tetrabutylammonium hydrogen sulphate (2.48 g, 7.24 mmol) were dissolved in methylene chloride (500 ml). To this was added a solution of sodium hydroxide (2.5 g, 140 mmol) in water (50 ml) and the mixture was heated at reflux for four
10 days, with daily additions of ethyl iodide (2.4 ml) and 20% sodium hydroxide (1 ml). After this time, no visible change in the composition of the reaction mixture could be detected by tlc (toluene-ethyl acetate 2:1). The mixture was allowed to cool, the phases separated and the organic phase washed
15 with water (3x) and dried over sodium sulphate. The solvent was removed under reduced pressure and the residue was subjected to flash chromatography on silica gel (toluene-ethyl acetate 6:1). The fractions containing the component with R_f = 0.40 on tlc (toluene-ethyl acetate 2:1) were pooled and re-
20 cromatographed as above to yield 3.15 g, 26% of the title compound. Fractions containing components with R_f = 0.32 on tlc were found to consist of a mixture of monoethylated monophenylmethylene acetal regio-isomers (^{1}H-NMR).

$[\alpha]_D^{20} = +11.2°(c1.02, CHCl_3)$

25 ^{1}H-NMR (CDCl$_3$, δ): 7.42 (symm.m, 2H), 6.88 (symm.m, 2H), 5.53 (s, 1H), 4.77 (d, 1.3 Hz, 1H, H1), 4.24 (dd, 4.2 Hz, 9.7 Hz, 1H, H6), 4.02 (dt, 4.0 Hz, 8.8 Hz, 1H, H5), 3.83 (dd, 1.8 Hz, 9.7 Hz, 1H, H6), 3.80-3.69 (s and overl.m, 6H, 1-OMe, H3,H4, 2-O-CHHCH$_3$), 3.66 (dd, 1.3 Hz, 3.7 Hz, 1H, H2), 3.64-3.55 (m,
30 1H, 2-O-CHHCH$_3$), 3.69 (s, 3H, Ar-OMe), 2.48 (d, 1H, OH), 1.26 (t, 7.0 Hz, 3H, 2-O-CH$_2$CH$_3$).

^{1}H-NMR (CDCl$_3$, with added Cl$_3$CCONC, δ): 8.54 (s, 1H, acylcarbamate NH), 7.38 (symm.m, 2H), 6.88 (symm.m, 2H), 5.52 (s, 1H), 5.27 (dd, 3.5 Hz, 10.3 Hz, 1H, H3), 4.76 (d, 1.6 Hz, 1H,

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H1), 4.32-4.20 (m, 1H), 4.13 (symm.m, 1H, H5), 3.97 (dd, 1.8
Hz, 3.5 Hz, 1H, H2), 3.91-3.80 (m, 1H), 3.79 (s, 3H, 1-OMe),
3.77-3.54 (m, 3H), 3.41 (s, 3H, Ar-OMe), 1.23 (t, 7.0 Hz,
3H).

5 ^{13}C-NMR (CDCl$_3$, δ): 160.1, 129.9, 127.6, 113.6, 102.0, 99.3,
79.4, 78.8, 68.7, 68.3, 67.2, 63.2, 55.2, 54.9, 15.4.

Calc. for C$_{17}$H$_{25}$O$_7$:   C: 59.8   H: 7.38,

*Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,6-O-(4'-me-
thoxy)phenylmethylene-α-D-mannohexopyranoside (bpy_9:2)*

10 Methyl 2-O-ethyl-4,6-O-(4'-methoxy)phenylmethylene-α-D-manno-
hexopyranoside (bpy_9:1) (0.98 g, 2.88 mmol) was dissolved in
dry methylene chloride (18 ml) together with triethyl amine
(600μl, 4.32 mmol) and the mixture was ice-cooled. t-Butyl-
dimethylsilyl trifluromethyl sulphonate (795 μl, 3.45 mmol)
15 in methylene chloride (2 ml) was added dropwise and the
reaction mixture with the cooling bath was left at ambient
temperature overnight (tlc silica toluene-methyl t-butyl
ether 4:1). The excess silylating reagent was destroyed by
addition of methanol (3 ml), the mixture was concentrated
20 under reduced pressure and the residue was subjected to flash
chromatography on silica (toluene-methyl t-butyl ether 40:1)
to yield 1.40 g, 100% of (bpy_9:2).

^{1}H-NMR (CDCl$_3$, δ): 7.40 (symm.m, 2H), 6.87 (symm.m, 2H), 5.53
(s. 1H), 4.70 (d, 1.3 Hz, 1H, H1), 4.21 (dd, 4.6 Hz, 9.9 Hz,
25 1H, H6), 4.10 (dd, 3.3 Hz, 9.7 Hz, 1H, H3), 3.91 (t, 9.5 Hz,
1H, H4), 3.90-3.79 (s overl. m, 5H, H6, 2-OCHHCH$_3$;1-OMe),
3.76-3.63 (m, 2H, H5, 2-OCHHCH$_3$), 3.55 (dd, 1.5 Hz, 3.3 Hz,
1H, H2), 3.67 (s, 3H, Ar-OMe), 1.24 (t, 7.0 Hz, 3H), 0.88 (s,
9H), 0.08 (s, 3H), 0.03 (s, 3H).

30 ^{13}C-NMR (CDCl$_3$, δ): 159.9, 130.3, 127.5, 113.4, 101.8, 101.2,
79.9, 79.1, 70.3, 68.8, 68.2, 64,1, 55.2, 54.8, 25.8, 18.3,
15.6, -4.4, -4.9.

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*Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-methoxy)-
benzyl-α-D-mannohexopyranoside (bpy_9:3)
and
methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-6-O-(4'-methoxy)-*
5 *benzyl-α-D-mannohexopyranoside (bpy_9:8)*

Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,6-O-(4'-me-
thoxy)phenylmethylene-α-D-mannohexopyranoside (bpy_9:2) (3.61
g, 7.95 mmol) was dissolved in acetonitrile (150 ml, dried
over 4 Å molecular sieves), powdered 3 Å molecular sieves (6
10 g) was added and the mixture cooled to -30°C under a nitrogen
atmosphere and stirred for 15 min. Sodium cyanoborohydride
(3.04 g, 47.7 mmol) was added and a solution of chlorotrime-
thylsilane (7.95 ml, 47.7 mmol) in dry acetonitrile (50 ml,
dried over 4 Å molecular sieves) was added dropwise in 1h.
15 Stirring was continued for 2h at -30°C, whereafter the tem-
perature was allowed to rise to 0°C. After 1h tlc (toluene-
ethyl acetate 4:1) showed complete conversion of starting
material. The mixture was filtered through a Celite® pad, the
Celite® washed thoroughly with ethyl acetate. The combined
20 filtrates were washed with aq. satd. sodium hydrogen carbo-
nate, aq. satd. sodium chloride, concentrated under reduced
pressure, redissolved in toluene and concentrated under
reduced pressure. The residue was subjected to flash chroma-
tography on silica (toluene-ethyl acetate 4:1, then 1:1) to
25 obtain bpy_9:8 (= bpy_21:1) 578 mg, 16% and (bpy_9:3) 2.34 g,
65%.

bpy_9:3 (some assignments from COSY, HETCOR):

^{1}H-NMR (CDCl$_3$, δ): 7.25 (symm.m 2H), 6.87 (symm.m, 2H), 4.81
(d, 11.0 Hz, 1H), 4.66 (d, 1.8 Hz, 1H, H1), 4.51 (d, 10.8 Hz,
30 1H), 4.02 (dd, 3.1 Hz, 5.9 Hz, 1H, H3), 3.80 (s, 3H, 1-OMe),
3.79-3.60 (m, 5H, H4, 2H6, 2-OCH$_2$CH$_3$), 3.54 (ddd, 2.9 Hz, 4.8
Hz, 9.7 Hz, 1H, H5), 3.46 (dd, 2.0 Hz, 3.3 Hz, 1H, H2), 3.33
(s, 3H, Ar-OMe), 2.10 and 1.86 (2 v.br.s, -1H, 6-OH), 1.23
(t, 6.8 Hz, 3H), 0.95 (s, 9H), 0.12 (s 6H).

^{1}H-NMR (CDCl$_3$, with added Cl$_3$CCONC, δ): 8.40 (s, 1H, acylcarbamate NH), 7.24 (symm.m, 2H, Ar H3',5'), 6.86 (symm.m, 2H, Ar H2',H6'), 4.82 (d, 11.2 Hz, 1H), 4.67 (d, 2.0 Hz, 1H, H1), 4.43-4.32 (m, 2H, 2H6), 4.08-3.98 (m, 1H, H3), 3.78 (s, 3H, 1-OMe), 3.77-3.61 (m, 4H, H5, 2-OCH$_2$CH$_3$, H4), 3.46 (dd, 1.8 Hz, 3.1 Hz, 1H, H2), 3.34 (s, 3H, Ar-OMe), 1.22 (t, 7.0 Hz, 3H), 0.96 (s, 9H), 0.14 (s, 3H), 0.13 (s, 3H).

^{13}C-NMR (CDCl$_3$, δ): 159.1, 130.7, 129.3, 113.8, 99.8, 79.5, 75.7, 74.7, 73.0, 72.2, 67.4, 62.5, 55.2, 54.7, 18.0, 15.7, -4.3, -4.6.

^{13}C-NMR (CDCl$_3$, with added Cl$_3$CCONC, δ): 159.3 (Ar C4'), 157.4, 149.3, 130.2 (Ar C2'), 129.6 (Ar C3',C5'), 113.8 (Ar C2',C6'), 99.6 (C1), 79.2 (C2), 74.56* (4-OCH$_2$Ar or 2-CH$_2$CH$_3$), 74.54* (4-OCH$_2$Ar or 2-CH$_2$CH$_3$), 73.2 (C3), 69.7 (C5), 67.2 (C4), 66.3 (C6), 55.2 (1-OMe), 54.9 (Ar-OMe), 25.9 (CMe$_3$), 18.0 (CMe$_3$), 15.6, -4.3, -4.7.

bpy_9:8:

^{1}H-NMR (CDCl$_3$, δ): 7.28 (symm.m, 2H), 6.86 (symm.m, 2H), 4.70 (d, 1.8 Hz, 1H, H1), 4.55 (d, 11.7 Hz, 1H, H6), 4.51 (d, 11.6 Hz, 1H, H6), 3.89-3.55 and 3.79 (m and s, 7H, H3, H4, H5, 2-OCH$_2$CH$_3$, 1-OMe), 3.44 (dd, 1.8 Hz, 2.9 Hz, 1H, H2), 3.56 (s, 3H, Ar-OMe), 2.29 (d, 2.0 Hz, 1H, 4-OH), 1.20 (t, 7.0 Hz, 3H), 0.92 (s, 9H), 0.13 (s, 3H), 0.12 (s, 3H).

^{13}C-NMR (CDCl$_3$, δ): 159.1, 130.3, 129.3, 113.7, 99.7, 78.9, 73.19*, 73.16*, 71.3, 70.3, 69.1, 67.2, 55.2, 54.8, 25.8, 18.2, 15.6, -4.5, -4.6.

*resolved by multiplying the FID with a gaussian weigth-function.

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Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-methoxy)-
benzyl-6(S)-phenyl-α-D-mannohexopyranoside (bpy_9:4)

(Oxidation ref. D.F. Taber *et al*, J. Org. Chem. 52, 5621-2,
1987) Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-

5   methoxy)benzyl-α-D-mannohexopyranoside (bpy_9:3) (200 mg,
0.438 mmol) and dimethylsulphoxide (64.0 μl, 0.876 mmol) were
dissolved in dichloromethane (2.5 ml, dried over 4 Å molecu-
lar sieves) and the solution was cooled on an ice-bath.
Phosphorpentoxide (124 mg, 0.876 mmol) was added quickly and

10   the resulting stirred suspension was allowed to attain RT
(1h). The mixture was again ice-cooled and triethyl amine
(215 μl, 1.53 mmol) was added causing an immediate disso-
lution of the gel-like suspension. The ice-bath was removed
and the mixture was stirred at RT for 1h, the diluted with

15   ethyl acetate, washed with aq. 0.2 M sodium dihydrogen phos-
phate, dried over sodium sulphate and concentrated under
reduced pressure (< 0.02 mbar). The residue was dissolved
tetrahydrofurane (2 ml, dried over 4 Å molecular sieves),
stirred and cooled to -20°C. Phenylmagnesium chloride in

20   tetrahydrofurane (345 μl of a 25 w% solution, 0.657 mmol,
Janssen, Belgium) was added dropwise (3 min). The reaction
was monitored with tlc (toluene-ethyl acetate 4:1) and
quenched with aq. 10% ammonium sulphate (3 ml). The mixture
diluted with ethyl acetate and washed twice with water, dried

25   over sodium sulphate and concentrated under reduced pressure.
The residue was subjected to flash chromatography on silica
(toluene-methyl t-butyl ether 20:1). The fractions containing
the component with a R_f = 0.44 on tlc (toluene-ethyl acetate
4:1) were pooled and concentrated under reduced pressure to

30   yield 65 mg, 30% of the title compound.

Molecular mechanics calculations with MM2(91) (Burkert and
Allinger, 1982) of the R and S diastereomers and subsequent
calculations with Karplus equation (Bothner-By, 1965) pre-
dicted that the ^{1}H-NMR $J_{5,6}$ would be 4.8 and 6.2 respective-

35   ly. This difference is too small to be but an indication of
the stereochemistry. Furthermore, the H6 signal is poorly

resolved and the H5 signal is severely overlapped by other
resonances. The ^{1}H-COSY H4-H5 crosspeak, however, contains
the sum $J_{4-5} + J_{5-6}$ and since the J_{4-5} is observable in the
H4-resonance, the J_{5-6} can be assigned the value 6.8 Hz. This
5 indicates that the isolated product has the 6-(S)
stereochemistry.

After the removal of the 4-methoxybenzyl group the calculated
coupling constants become 5.5 Hz and 12.9 Hz, respectively.

^{1}H-NMR (CDCl$_3$, δ): 7.44-7.19 (m, 7H), 6.89 (symm.m, 2H), 5.00
10 (br.m, 1H, H6), 4.90 (d, 10.8 Hz, 1H), 4.66 (d, 10.8 Hz, 1H),
4.58 (d, 1.8 Hz, 1H, H1), 4.04 (dd, 3.1 Hz, 9.0 Hz), 3.99 (t,
9.2 Hz, 1H, H4), 3.81 and 3.82-3.56 (s and m, 6H, 1-OMe, 2-
CH$_2$CH$_3$), H5), 3.44 (dd, 2.0 Hz, 2.9 Hz, 1H, H2), 2.84 (s, 3H,
Ar-OMe), 1.24 (t, 3H), 1.97 (s, 9H), 0.15 (s, 3H), 0.13 (s,
15 3H).

^{13}C-NMR (CDCl$_3$, δ): 159.0, 142.5, 130.8, 129.2, 127.7, 126.7,
126.6, 125.7, 113.7, 99.5, 79.8, 76.1, 74.5, 73.0, 70.9,
67.9, 55.1, 54.1, 25.8, 17.9, 15.4, -4.5, -4.8.

Methyl 2,3-anhydro-4,6-O-p-methoxybenzylidene-α-D-mannopyra-
20 *noside (bpy_9:9)*

To a stirred suspension of sodium hydride (2.6 g, 65 mmol,
65% dispersion in mineral oil) in N,N-dimethylformamide (150
ml) was added with stirring a solution of methyl 4,6-O-p-
methoxybenzylidene-α-D-glucopyranoside (9.36 g, 30 mmol) in
25 N,N-dimethylformamide (65 ml). The mixture was stirred to 45
minutes and p-toluenesulfonylimidazol (7.24 g, 33 mmol was
added. Stirring was continued for 2 hours and the mixture was
the poured into ice-water. The precipitate was filtered of,
dried in vacuo to give crude bpy_9:) (7.7 g).
30 Recrystallization twice form methanol (250 ml gave bpy_9:9
(3.58 g). Chromatography (SiO$_2$, ethyl acetate-heptane, 2:3)
of the mother liquor followed by crystallization form me-

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thanol (150 ml) gave additional bpy_9:9 (1.87 g). The total
yield of bpy_9:9 was 5.45 g (61%).

m.p.   152-153.5°C (methanol)
$[\alpha]_D^{22}$: 96.4° (c 1.8, chloroform)

5   ^{1}H NMR (CDCl$_3$) δ: 7.43 and 6.91 (AB pattern, further coupled,
2H, J_{AB}=8.7 Hz), 5.53 (s, 1H, ArCH), 4.90 (s, 1H, H-1), 4.30-
4.19 (sym.m, 1H) 3.82 (s, 3H, CH$_3$OAr), 3.78-3.64 (3H), 3.49-
3.46 (4H), 3.48 (s, 3H, CH$_3$O), 3.18 (d, 1H, J 3.7=Hz, H-2/H-
3) ppm.

10   ^{13}C NMR (CDCl$_3$) δ: 160.3, 129.6, 127.5, 113.7, 102.4, 96.9,
74.8, 69.4, 61.7, 55.7, 55.3, 53.8 and 50.5 ppm.

*Methyl 3-azido-4,6-O-p-methoxybenzylidene-α-D-altropyranoside
(bpy_9:10)*

Methyl 2,3-anhydro-4,6-O-p-methoxybenzylidene-α-D-mannopyra-
15   noside (bpy_9:9, 2.35 g, 8 mmol), sodium azide (2.08 g, 32
mmol) and ammonium chloride (0.86 g, 16 mmol) was stirred for
5 hours at 110°C in a mixture of 2-methoxyethanol (25 ml) and
water (5 ml). The suspension gradually dissolved to give a
turbid solution. The mixture was then partitioned between
20   ethyl acetate and aqueous NaOH (0.25 M, 100 ml). The aqueous
phase was extracted twice with ethyl acetate and the combined
organic phases was washed twice with water and than with
saturated aqueous NaCl, dried (Na$_2$SO$_4$), filtered and concen-
trated. Chromatography (SiO$_2$) ethyl acetate-heptane, 2:3-2:1)
25   gave bpy_9:10 (1.97 g, 73%). An analytical sample was crys-
tallized from ethyl acetate-heptane.

m.p.   91-93°C (EtOAc/Heptane)
$[\alpha]_D^{22}$: +26.1° (c 1.1, chloroform)

^{1}H NMR (CDCl$_3$) δ: 7.44 and 6.91 (AB pattern further coupled,
30   2H, J_{AB}=8.6 Hz), 5.57 (s, 1H, ArCH), 4.56 (s, 1H, H-1), 4.43-
4.21 (2H), 4.16-4.08 (1H), 4.03 (unresolved dd, 1H, J_1=J_2=3

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Hz), 3.91 (bs, 1H, H-2), 3.83-3.74 (4H), 3.80 (s, 3H, CH₃O), 3.43 (s, 3H, CH₃O), 2.36 (bs, 1H, OH) ppm.

^{13}C NMR (CDCl₃) δ: 160.3, 129.5, 127.5, 113.7, 102.3, 101.4, 75.8, 69.8, 69.0, 60.1, 59.0, 55.7 and 55.3 ppm.

5   Acetylation (acetic anhydride-pyridine, 3:5) of a sample of bpy_9:10 gave an acetate which had the following ^{1}H NMR spectrum (assignments were confirmed by the corresponding homonuclear COSY spectrum):

^{1}H NMR (CDCl₃) δ: 7.43 and 6.91 (AB pattern further coupled,
10   2H, J_{AB}=8.8 Hz), 5.59 (s, 1H, ArCH), 4.97 (dd, 1H, J=2.2 and 0.9 Hz, H-2), 4.57 (s, 1H, H-1), 4.36 (2H, H-6 and H-4), 4.09-4.01 (2H, H-6, and H-3), 3.84-3.74 (2H, CH₃O and H-5), 3.81 (s, 3H, CH₃O), 3.44 (s, 3H, CH₃O), 2.15 (s, 3H, CH₃CO) ppm.

15   *Methyl 3-azido-2-O-ethyl-4,6-O-p-methoxybenzylidene-α-D-altropyranoside (bpy_9:11)*

Methyl 3-azido-4,6-O-p-methoxybenzylidene-α-D-altropyranoside (bpy_9:10, 1.7 g, 5 mmol), barium oxide (3.0 g, 19.6 mmol) and ethyl iodide (5 ml, 62 mmol) was stirred in dimethyl
20   sulphoxide (5 ml). Water (10 μl) was added and the stirring was continued for 16 hours. The mixture was then partitioned between ethyl acetate and water. The aqueous phase was extracted twice with ethyl acetate and the combined organic phases was washed twice with water and then with saturated
25   aqueous NaCl, dried (Na₂SO₄), filtered and concentrated. Chromatography (SiO₂, ethyl acetate-heptane, 1:3) gave bpy_9:11 as an oil (1.7 g, 92%).

$[\alpha]_D^{22}$: 19.1° (c 1.6, chloroform).

^{1}H NMR (CDCl₃) δ: 7.44 and 6.91 (AB pattern, further coupled,
30   2H, J_{AB}=8.6 Hz), 5.57 (s, 1H, ArCH ...

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(sym.m.2H), 3.52 (dd, 1H, J=2.4 and 0.9 Hz, H-3), 3.34 (s, 3H, CH$_3$O), 1.25 (t, 3H, J=6.9 Hz, CH$_3$CH$_2$) ppm.

^{13}C NMR (CDCl$_3$) δ: 160.1, 129.6, 127.4, 113.6, 102.1, 99.6, 77.0, 76.1, 69.0, 66.5, 58.7, 58.1, 55.5, 55.2 and 15.3 ppm.

5   *Methyl 3-azido-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-α-D-altro-pyranoside*

(Cf. R. Johansson and B. Samuelsson; *J. Chem. Soc. Commun.* 1984, 201-202).

Methyl 3-azido-3-deoxy-2-O-ethyl-4,6-O-p-methoxybenzylidene-
10   α-D-altropyranoside (bpy_9:11) (2.41 g, 6.6 mmol), sodium cyanoborohydride (2.5 g, 39.6 mmol) and ground molecular sieves (5 g) was stirred in acetonitrile (130 ml) at 0°C. A solution of trimethylsilyl chloride (5.0 ml, 40 mmol) in acetonitrile (45 ml) was added during 55 min. After addi-
15   tional 1.5 h of stirring, the cooling bath was removed and the stirring was continued for 21 h. The mixture was filtered through celite and the filtrate was partitioned between ethyl acetate and saturated aqueous NaHCO$_3$. The organic phase was washed twice with saturated aqueous NaHCO$_3$ and then with
20   saturated aqueous NaCl, dried (Na$_2$SO$_4$+NaHCO$_3$), filtered and evaporated. Chromatography twice (SiO$_2$, ethyl acetate-heptane 2:7→2:1 and ethyl acetate-toluene 1:2→2:1) gave the title compound (2.23 g, 92 %) as an oil.

$[\alpha]_D^{22}$: +113.6° (c 1.4, chloroform)

25   ^{1}H NMR data (CDCl$_3$) δ: 7.30 and 6.89 (AB pattern, further coupled, 4 H, J - 8.8 Hz), 4.62 and 4.56 (AB pattern, 2 H, J = 11.3 Hz, benzylic H), 4.59 (unresolved d, virtually coupled, 1 H, J <2 Hz, H-1), 4.00-3.84 (3 H, H-3, H-4 and H-5), 3.81 (s, 3 H, CH$_3$OAr), 3.84-3.7 (2 H, H-6), 3.62-3.43 (3
30   H, CH$_2$CH$_3$ and H-2), 3.38 (s, 3 H, CH$_3$O), 1.99 (bs, 1 H, OH) and 1.19 (t, 3 H, J = 7.0 Hz, CH$_2$CH$_3$) ppm.

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^{13}C NMR data (CDCl$_3$) δ: 159.6, 129.9, 129.6, 113.9, 99.9, 76.9, 72.2, 71.9, 67.7, 66.3, 62.4, 58.6, 55.4, 55.3 and 15.4 ppm.

Methyl 3-azido-6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxy-benzyl-α-D-altropyranoside

A solution of methyl 3-azido-3-deoxy-2-O-ethyl-4-O-p-methoxy-benzyl-α-D-altro-pyranoside (1.47 g, 4 mmol) in pyridine (35 ml) was stirred at 0°C. Benzoyl chloride (1.2 ml, 10.3 mmol) was added and the temperature was raised to room temperature. The stirring was continued for 1.5 h and the mixture was then cooled to 0°C. Methanol (10 ml) was added and the mixture was stirred for additional 20 min. at room temperature, evaporated and partitioned between ethyl acetate and water. The organic phase was washed subsequently with water, saturated aqueous NaHCO$_3$, water, saturated aqueous NaCl, dried (Na$_2$SO$_4$), filtered and evaporated. Chromatography (SiO$_2$, ethyl acetate-heptane 1:4) gave the benzoate (1.64 g, 87%).

$[\alpha]_D^{22}$: +0.95° (c 1.0, chloroform)

^{1}H NMR data (CDCl$_3$) δ: 8.04-7.98 (m, 2 H), 7.56 (tt, 1 H, J = 7.5 and 1.3 Hz), 7.43 (t, further coupled, 2 H, J = 7.5 Hz), 7.26 and 6.83 (AB pattern, further coupled, 4 H, J = 8.6 Hz), 4.62 and 4.50 (AB pattern, 2 H, J = 11.3 Hz, ArCH$_2$), 4.63 (d, further coupled, J = 1.3 Hz, H-1), 4.54 (A part of an ABX system, 1 H, J = 11.8 and 2.7 Hz, H-6$_a$), 4.47 (B part of an ABX system, 1 H, J = 11.8 and 5.5 Hz, H-6$_b$), 4.27 (ddd, 1 H, J = 8.4, 5.5 and 2.7 Hz, H-5), 3.99-3.92 (2 H, H-3 and H-4), 3.74 (s, 3 H, CH$_3$OAr), 3.67-3.48 (3 H, CH$_2$CH$_3$ and H-2) and 1.22 (t, 3 H, J = 7.0 Hz, CH$_2$CH$_3$) ppm.

^{13}C NMR data (CDCl$_3$) δ: 166.2, 159.5, 132.9, 130.1, 129.9, 129.6, 129.2, 128.3, 113.9, 99.6, 76.72, 72.0, 71.7, 62.3, 62.2, 64.1, 58.5, 55.4, 55.2 and 15.4 ppm.

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Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-sulfamino-α-D-altropyranoside sodium salt

(Cf. H. P. Wessel; *J. Carbohydr. Chem.* 11 (8), (1992), 1039-1052).

5   To a stirred solution of methyl 3-azido-6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-α-D-altropyranoside (620 mg, 1.31 mmol) in tetrahydrofuran (40 ml) was added water (265 ml) and triphenylphosphine (1.7 g, 6.5 mmol). The solution was stirred for 24 h and was then concentrated to 12 ml. The
10   residue was diluted with methanol (10 ml) and water (3 ml). To the stirred solution was then added trimethylamine sulfurtrioxide complex (370 mg, 2.7 mmol). The pH was adjusted to 8.5 by 1 M aqueous NaOH. After a few minutes a precipitate was formed, which was dissolved by adding tetrahydrofuran (3
15   ml) and methanol (4 ml). The reaction mixture was stirred for 0.5 h, after which time additional trimethylamine sulfurtri-oxide complex (86 mg) was added. The stirring was continued for 15 min. During the course of the reaction pH was kept at 8-9 by adding 1 M aqueous NaOH. The mixture was then diluted
20   with water (7 ml) and the organic solvents were evaporated under reduced pressure. Additional water (5 ml) was added and the suspension was lyophilised and chromatographed (SiO$_2$, chloroform-methanol-water 100:15:1→70:30:5) to give the sulfamino compound, presumably in the sodium salt form, (668
25   mg, 92 %) after lyophilisation from water. A sample was crystallized from aqueous acetone.

m.p. 112-115°C (aqueous acetone).
$[\alpha]_D^{22}$: +156.4° (c 0.5, water)

^{1}H NMR data (CD$_3$OD) δ: 7.94-7.89 (m, 2 H), 2 H, 7.64-7.56 (m,
30   1 H), 7.44 (m, 2 H), 7.28 and 6.73 (AB pattern, further coupled, 4 H, J = 8.6 Hz), 4.71 and 4.49 (AB pattern, 2 H, J = 11.4 Hz, ArCH$_2$), 4.68 (bs, 1 H, H-1), 4.51 (dd, 1 H, J = 11.6 and 2.4 Hz, H-6$_a$), 4.44 (dd, 1H, J = 11.6 and 4.4 Hz, H-6$_b$), 4.09 (unresolved ddd, 1 H, J = 4, 3 and 1 Hz, H-3),

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4.00-3.92 (2 H, H-5 and H-2), 3.86 (dd, 1 H, J = 9.9 and 4.2 Hz, H-4), 3.71-3.54 (5 H, CH_2CH_3 and CH_3OAr), 3.65 (s, CH_3OAr), 3.40 (s, 3 H, CH_3O) and 1.17 (t, 3 H, J = 7.0 Hz, CH_2CH_3) ppm.

5   ^{13}C NMR data (CD_3OD) δ: 167.8, 160.8, 134.2, 131.4, 130.9, 130.6, 129.5, 114.7, 101.6, 77.03, 70.02, 69.2, 67.0, 66.4, 65.2, 55.6, 55.5, 50.6 and 15.8 ppm.

Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-3-sulfamino-α-D-altropyranoside ammonium salt (bpy_30)

10   Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-sulfamino-α-D-altro-pyranoside sodium salt (334 mg, 2.0 mmol) was hydrogenolysed for 6 h in glacial acetic acid (55 ml) at 0.23 MPa and room temperature, using 5 % palladium on char-coal as a catalyst. Filtering and lyophilisation gave a
15   residue which was chromatographed (SiO₂, chloroform-methanol-water 100:15:1→70:30:5). The product was dissolved in water and passed through a cation exchange resin (BIO-REX® 70, 200-400 mesh, ammonium form) and lyophilised to give the title compound (222 mg, 86 %)

20   $[\alpha]_D^{22}$: +55.7° (c 1.3, water)

^{1}H NMR data (pyridine-d₅) δ: 8.23-8.17 (2 H), 7.52-7.45 (1 H), 7.42-7.35 (2 H), 5.09 (dd, 1 H, J = 11.65 and 1.54 Hz, H-6ₐ), 4.82 (dd, 1 H, J = 11.43 and 6.81 Hz, H-6ᵦ), 4.82 (s, 1 H, H-1), 4.66 (unresolved dd, 1 H, J ≈ 3.5 and 3.5 Hz, H-3),
25   4.36 (dd, 1 H, J = 10.11 and 3.96 Hz, H-4), 4.32-4-23 (1 H, H-5), 4.18 (d, 1 H, J = 3.08 Hz, H-2), 3.60-3.44 (2 H, CH_2CH_3), 3.16 (s, 3 H, CH_3O) and 1.00 (t, 3 H, J = 7.0 Hz, CH_2CH_3) ppm.

^{1}H NMR data (D_2O, ref. acetone at 2.35 ppm) δ: 8.22-8.17 (2
30   H), 7.83 (tt, 1 H, J = 7.5 and < 2 Hz), 7.72-7.65 (2 H), 4.97 (HDO), 4.93 (s, 1 H, H-1), 4.81 (dd, virtually coupled, 1 H, J = 12 and 1.7 Hz, H-6ₐ), 4.65 (dd, virtually coupled, 1 H, J

= 12 and 5.5 Hz, H-6$_b$), 4.18-4-15 (2 H, h-4 and H-5), 4.05
(dd, 1 H, J = 3.3 and 1.4 Hz, H-2), 3.95-3.75 (sym. m. 2 H,
C$\underline{H}_2$CH$_3$), 3.94-3.91 (m, 1 H, H-3), 3.54 (s, 3 H, CH$_3$O) and
1.35 (t, 3 H, J = 7 Hz, CH$_2$C$\underline{H}_3$) ppm (the assignments of the
^{1}H NMR spectrum signals were made on the basis of a COSY
experiment).

^{13}C NMR data (D$_2$O, ref.: acetone at 33.19 ppm) δ171.4, 136.9,
132.5, 132.1, 131.7, 102.5, 78.4, 69.9, 69.1, 67.4, 65.7,
58.1, 56.0 and 17.5 ppm.

*Methyl 3-azido-6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxy-
benzyl-α-D-altropyranoside*

A solution of methyl 3-azido-3-deoxy-2-O-ethyl-4-O-p-methoxy-
benzyl-α-D-altro-pyranoside (476 mg, 1.3 mmol) in pyridine (8
ml) was stirred at 0°C. Pivaloyl chloride (390 µl, 3.25 mmol)
was added and the temperature was raised to room temperature.
The stirring was continued for 1.5 h and the mixture was then
cooled to 0°C. Methanol (10 ml) was added and the mixture was
stirred for additional 20 min. at room temperature, evapo-
rated and partitioned between ethyl acetate and water. The
organic phase was washed subsequently with water, saturated
aqueous NaHCO$_3$, water, saturated aqueous NaCl, dried
(Na$_2$SO$_4$), filtered and evaporated. Chromatography (SiO$_2$,
methyl tert-butyl ether-heptane 1:4) and (C-18 Lobar, Merck,
acetonitrile-water 4:1) gave the pivaloate (431 mg, 75 %).

^{1}H NMR data (CDCl$_3$) δ: 7.28 and 6.89 (AB pattern, further
coupled, 4 H, J = 8.6 Hz), 4.60 and 4.49 (AB pattern, 2 H, J
= 11.0 Hz, ArC$\underline{H}_2$), 4.57 (s, 1H, H-1), 4.39 (dd, 1 H, J = 14.5
and 5.3 Hz, H-6$_a$), 4.13 (2 H, H-6$_b$ and H-4), 3.93 (m, 1 H, H-
3), 3.84-3.78 (4 H, H-5 and MeOAr), 3.64-3.45 (3 H, C$\underline{H}_2$CH$_3$
and H-2), 3.39 (s, 3H, 1-OMe) and 1.22-1.15 (s and t, 12 H, J
= 7.0 Hz, C(CH$_3$)$_3$ and CH$_2$C$\underline{H}_3$) ppm.

^{13}C NMR data (CDCl$_3$) δ: 178.1, 159.6, 129.9, 129.3, 113.9,
99.5 (C-1), 76.7 (C-2), 72.5 (C-5), 71.9 (Ar$\underline{C}$H$_2$), 66.4 (C-4),

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66.2 ($\underline{C}H_2CH_3$), 63.6 (C-6), 58.5, 55.3, 55.2, 38.8, 27.2 and
15.4 ppm.

*Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
sulfamino-α-D-altropyranoside sodium salt*

5   (Cf. H. P. Wessel; *J. Carbohydr. Chem.* 11 (8), (1992), 1039-
     1052.)

     To a stirred solution of methyl 3-azido-6-O-pivaloyl-3-deoxy-
     2-O-ethyl-4-O-p-methoxybenzyl-α-D-altropyranoside (300 mg,
     0.683 mmol) in tetrahydrofuran (20 ml) was added water (150
10   ml) and triphenylphosphine (0.90 g, 3.3 mmol). The solution
     was stirred for 24 h and was then concentrated to 5 ml. The
     residue was diluted with methanol (5 ml) and water (1 ml).
     One half of this solution was used to prepare the correspon-
     ding oxamate (see 48.182). To the remaining half of the
15   stirred solution (0.342 mmol) was then added trimethylamine
     sulfurtrioxide complex (99 mg, 0.719 mmol). The pH was
     adjusted to 8.5 by 1M aqueous NaOH. After a few minutes a
     precipitate was formed, which was dissolved by adding tetra-
     hydrofuran (0.7 ml) and methanol (1 ml). The reaction mixture
20   was stirred for 0.5 h, after which time additional trimethyl-
     amine sulfurtrioxide complex (45 mg) was added The stirring
     was continued for 15 min. During the course of the reaction
     pH was kept at 8-9 by adding 1M aqueous NaOH. The mixture was
     then diluted with water (2 ml) and the organic solvents were
25   evaporated under reduced pressure. Additional water (5 ml)
     was added and the suspension was lyophilised and chromato-
     graphed (SiO$_2$, chloroform-methanol-water 100:15:1→80:20:1) to
     give the sulfamino compound, presumably in the sodium salt
     form, (161 mg, 80 %) after lyophilisation from water.

30   ^{1}H NMR data (dmso-D$_6$) δ: 7.24 and 6.87 (AB pattern, further
     coupled, 4 H, J = 8.8 Hz), 4.69 and 4.24 (AB pattern, 2 H, J
     = 10.8 Hz, ArCH$_2$), 4.56 (s, 1H, H-1), 4.29 (dd, 1 H, J = 11.4
     and 1.7 Hz, H-6$_a$), 4.05-3.96 (2 H, H-6$_b$ and NH), 3.83-3.76
     (m, 2 H, H-2 and H-3), 3.76-3.73 (4 H, s and m, H-5 and

MeOAr), 3.54-3.40 (3 H, $C\underline{H}_2CH_3$ and H-4), 3.27 (s, 3H, 1-OMe) and 1.12-1.02 (s and t, 12 H, J = 7.0 Hz, $C(CH_3)_3$ and $CH_2C\underline{H}_3$) ppm.

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-3-sulfamino-α-D-altro-
5 *pyranoside ammonium salt (bpy_37)*

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-sulfamino-α-D-altro-pyranoside sodium salt (153 mg, 0.259 mmol) was hydrogenolysed for 6 h in glacial acetic acid (8 ml) at 0.23 MPa and room temperature, using 5 % palladium on
10 charcoal as a catalyst. Filtering and lyophilisation gave a residue which was chromatographed (SiO_2, chloroform-methanol-water 80:20:1 with 0.1% NH_3) to give the title compound (77.2 mg, 74 %).

^{1}H NMR data (D_2O, ref. acetone at 2.35 ppm) δ: 4.83 (HDO),
15 4.78 (s 1 H, H-1), 4.43 (dd, virtually coupled, 1 H, J = 11.5 and 1.1 Hz, H-6_a), 4.30 (dd, virtually coupled, 1 H, J = 11.5 and 5.1 Hz, H-6_b), 3.95-3.91 (2 H, H-4 and H-5), 3.89 (dd, 1 H, J = 3.3 and 1.3 Hz, H-2), 3.80-3.62 (m. 3 H, H-3 and $C\underline{H}_2CH_3$), 3.42 (s, 3 H, CH_3O) and 1.24-1.21 (s and t, 12 H, J
20 = 7 Hz, [t]Bu and $CH_2C\underline{H}_3$) ppm (the assignments of the ^{1}H NMR spectrum signals were made on the basis of a COSY experiment).

^{13}C NMR data (D_2O, ref.: acetone at 33.19 ppm) δ188.3, 106.2,
82.2, 73.8, 72.8, 70.6, 69.3, 61.9, 59.8,45.6, 37.1 and 21.3
25 ppm.

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
[t]butyloxamido-α-D-altropyranoside

Oxalyl chloride (120 μl, 1.37 mmol) dissolved in tetrahydro-furane (1 ml) was cooled to -26°C. A solution of t-butanol
30 (131 mg, 1.77 mmol) and pyridine (154 μl, 1.91 mmol) in tetrahydrofurane (2 ml) was added dropwise and the mixture

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was stirred at -26°C for 15 min, while the initially formed
yellowish precipitate turned white.

The solvents were removed under reduced pressure from the
tetrahydrofurane solution from the reduction of 3-azido-6-O-
5   pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-α-D-altropyra-
noside (150 mg, 0.342 mmol). The residue was redissolved in
tetrahydrofurane (2.5 ml) and pyridine (154 μl) and added
dropwise to the cooled solution above and stirred at -26°C
for 1h. Water (2 ml) was added and the mixture diluted with
10   t-butyl methyl ether (15 ml) and washed with water, saturated
sodium hydrogen carbonate and brine. The organic solution was
dried over sodium sulfate and the solvents were removed under
reduced pressure. The residue was chromatographed (SiO$_2$, t-
butyl methyl ether - toluene 1:8) to yield 187 mg, 100% of
15   the title compound.

^{1}H NMR data (CDCl$_3$) δ: 8.25 (d, 1H, 10.1 Hz, 3-NH), 7.26 and
6.85 (AB pattern, further coupled, 4 H, J = 8.6 Hz), 4.82-
4.71 (m and d, 2H, 10.5 Hz, H-3 and ArCH$_2$), 4.69 (s, 1H, H-
1), 4.42 (dd, 1H, 1.7 and 11.7 Hz, H-6$_a$) 4.31 (d, 1H, 10.5
20   Hz, ArCH$_2$), 4.16 (dd, 1H, 6.2 and 11.7Hz, H-6$_b$), 3.88 (ddd,
1H, 1.8, 5.9 and 10.3 Hz, H-5), 3.81-3.75 (dd and s, 4H, H-4
and ArOMe), 3.64-3.49 (m, 2H, CH$_2$CH$_3$), 3.47-3.43 (dd and s,
4H, 1.3 and 3.1 Hz, H-2 and 1-OMe), 1.55 (s, 9H, oxamate
C(CH$_3$)$_3$), 1.19 and 1.18 (s and t, 12H, 7 Hz, 6-O pivaloate
25   ^{t}Bu and CH$_2$CH$_3$) ppm.

^{13}C NMR data (CDCl$_3$) δ: 178.1, 159.4, 159.1, 157.4, 130.3,
129.4, 113.8, 98.9, 84.2, 77.2, 75.9, 70.8, 69.5, 65.8, 65.7,
63.4, 55.2, 55.1, 45.7, 38.8, 27.7, 27.1, 15.3 ppm.

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-3-oxamido-α-D-altro-
30   *pyranoside ammonium salt (bpy_54)*

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
^{t}butyloxamido-α-D-altropyranoside (180 mg, 332 μmol) was
dissolved in acetic acid-ethanol mixture (1:1, 6 ml),
palladium on carbon (5%, 250 mg) was added and subjected for

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hydrogenolysis at 39 psi overnight. The catalyst was filtered
of on a celite pad, washed with ethanol. The filtrate was
concentrated under reduced pressure, redissolved in dichloro-
methane (2.4 ml). Trifluoroacetic acid (960 μl) was added and
5   the mixture stirred for 4.5h at room temperature. Water (3
ml) and ammonia (3M, 300 μl) was added and reaction mixture
was concentrated under reduced pressure and lyophilized. The
residue was chromatographed (SiO$_2$, chloroform-methanol-water
80:20:1 with 0.1% NH$_3$) to give the title compound, 117.1 mg,
10 89%.

^{1}H NMR data (D$_2$O, ref. acetone at 2.35 ppm) δ: 4.80 (HDO),
4.78 (s 1 H, H-1), 4.45-4.35 (m, 2H, H-3 and H-6$_a$), 4.26 (dd,
1H, 4.4 and 12.1 Hz, H-6$_b$), 4.07-3.96 (m, 2H, H-4 and H-5),
3.77-3.58 (m, 3H, CH$_2$CH$_3$ and H-2), 3.45 (s, 3H, 1-OMe), 1.20-
15 1.13 (s and t, 12 H, 6-O-pivaloate ^{t}Bu and CH$_2$CH$_3$) ppm.

^{13}C NMR data (D$_2$O, ref.: acetone at 33.19 ppm) δ: 188.1,
171.9, 171.4, 105.7, 82.4, 73.7, 72.9, 70.3, 69.7, 62.0,
56.1, 45.9, 37.0, 21.2 ppm.

Methyl 3-azido-6-O-pyrrol-3'-ylcarboxyl-3-deoxy-2-O-ethyl-4-
20 *O-p-methoxybenzyl-α-D-altropyranoside*

A solution of methyl 3-azido-3-deoxy-2-O-ethyl-4-O-p-methoxy-
benzyl-α-D-altro-pyranoside (476 mg, 1.3 mmol) in pyridine
(496 μl) and dichloromethane (2 ml) was stirred at 0°C.
Methanesulfonyl chloride (380 μl, 4.90 mmol) was added and
25 the temperature was raised to room temperature. The stirring
was continued for 5 h and the mixture was then cooled to 0°C.
Water (1 ml) was added and the mixture was stirred for addi-
tional 20 min. at room temperature, evaporated and parti-
tioned between ethyl acetate and water. The organic phase was
30 washed subsequently with water, saturated aqueous NaHCO$_3$,
water, saturated aqueous NaCl, dried (Na$_2$SO$_4$), filtered and
evaporated. Chromatography (SiO$_2$, methyl tert-butyl ether-
toluene 1:9) yielded 216 mg (79%) of the mesylate as a yel-
lowish oil.

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N-Triisopropylsilyl pyrrole-3-carboxylic acid (484 mg, 1.81 mmol) and 1,8-diazacicyclo[5.4.0]undec-7-ene (255 µl) were dissolved in dimethylformamide (1 ml) and added to a solution of the mesylate above in dimethylformamide (1 ml).
5 The mixture was heated at 90°C overnight and the subjected to chromatography (210 g C-8 Lobar, Merck, acetonitrile-water 3:2) to yield 30 mg, 16 % of the pyrroloyl ester.

^{1}H NMR data (CDCl$_3$) δ: 8.73 (br.s., 1H, pyrrole NH), 7.39 (symm. m., 1H, pyrrole H-4), 7.27 and 6.85 (AB pattern,
10 further coupled, 4 H, J = 8.8 Hz), 3.74 (symm. m, 1H, pyrrole H-2), 3.64 (symm. m, 1H, pyrrole H-5), 4.63-4.49 (s and 2 d, 3H, H-1 and ArCH$_2$), 4.48-4.36 (m, 2H, 2 H-6), 4.22 (symm. m, 1H, H-5), 3.94-3.84 (m, 2H, H-3 and H-4), 3.78 (s, 3H, ArOMe), 3.68-3.55 (m, 3H, CH$_2$CH$_3$ and H-2), 3.40 (s, 3H, 1-
15 OMe), 1.20 (t, 3H, 6.8 Hz, CH$_2$CH$_3$) ppm.

^{13}C NMR data (CDCl$_3$) δ: 164.6, 159.5, 129.9, 129.4, 123.6, 11.7, 116.1, 113.9, 109.9, 99.9, 76.9, 72.5, 72.0, 66.8, 66.3, 63.0, 58.9, 55.3, 55.2, 15.4 ppm.

Methyl 6-O-pyrrol-3'-ylcarboxyl-3-deoxy-2-O-ethyl-3-sulfami-
20 *no-α-D-altropyranoside ammonium salt (bpy_40)*

To a stirred solution of methyl 3-azido-6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-α-D-altropyranoside (30 mg, 61 µmol) in tetrahydrofuran (1 ml) was added water (11 µl) and triphenylphosphine (83 mg, 305 µmol). The solution was
25 stirred for 24 h, diluted with and water (1 ml). Trimethyl-amine sulfurtrioxide complex (9 mg, 0.72 µmol) was then added. The pH was adjusted to 8.5 by 1 M aqueous NaOH. After a few minutes a precipitate was formed, which was dissolved by adding tetrahydrofuran (0.7 ml) and methanol (1 ml). The
30 reaction mixture was stirred for 0.5 h, after which time additional trimethylamine sulfurtrioxide complex (9 mg) was added. The stirring was continued for 15 min. During the course of the reaction pH was kept at 8-9 by adding 1M aqueous NaOH. The mixture was then diluted with water (2 ml),

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washed with t-butyl methyl ether, lyophilised and chromato-
graphed (SiO$_2$, chloroform-methanol-water 80:20:1) to give the
sulfamino compound, presumably in the sodium salt form, (30.6
mg, 93 %) after lyophilisation from water.

5   ^{1}H NMR data (CD$_3$OD) δ: 7.35 (unresol. dd, x part of a ABX-
pattern, 1H, J_{AX}+J_{BX}=3.5 Hz, pyrrole H-2), 7.29 and 6.79 (AB
pattern, further coupled, 4 H, J = 8.6 Hz), 6.75 (dd, 1H, 2.0
and 2.9 Hz, pyrrole H-4), 6.51 (dd, 1H, 1.3 and 2.9 Hz,
pyrrole H-5), 4.71 and 4.46 (AB pattern, 2 H, J = 11.4 Hz,
10   ArCH$_2$), 4.66 (bs, 1 H, H-1), 4.39 and 4.33 (2 dd, 2H, 2.6,
5.1 and 11.7 Hz, 2 H-6), 4.04 (m, 1H, H-3), 3.97-3.87 (m and
dd, 2H, 1.1 and 3.1 Hz, H-5 and H-2), 3.80 (dd, 1H, 4.4 and
10.1 Hz, H-4) 3.73 (s, 3H, ArOMe), 3.68-3.52 (m, 2H, CH$_2$CH$_3$),
3.39 (s, 3H, 1-OMe), 1.15 (t, 3H, 7.0Hz, CH$_2$CH$_3$) ppm.

15   ^{13}C NMR data (CD$_3$OD) δ: 167.32, 160.9, 131.3, 131.0 125.3,
120.1, 116.3, 114.7, 110.2, 101.7, 77.2, 10.4, 69.9, 67.2,
66.4, 55.6, 55.5, 50.9 and 15.8 ppm.

The residue was dissolved in methanol (15 ml), palladium on
carbon (5%, 50 mg) was added and the mixture hydrogenolyzed
20   at 30 psi for 3 h. The catalyst was filtered off, the fil-
trate concentrated under reduced pressure, redissolved in
ammonia (2%) in methanol-water (1:1, 0.6 ml) and chromato-
graphed (3 ml C-18 Supelco, methanol-water 2:3). Methanol was
removed under reduced pressure from the pooled product-con-
25   taining fractions and the residue lyophilized to give 6.2 mg
of the title compound as the ammonium salt.

^{1}H NMR data (CD$_3$OD) δ: 7.35 (m, 1H, pyrrole H-2), 6.75 (dd,
1H, 2.0 and 2.9 Hz, pyrrole H-4), 6.55 (dd, 1H, 1.3 and 2.9
Hz, pyrrole H-5), 4.67 (br.s, 1H, H1), 4.55 (dd, 1H, 1.3 and
30   11.4 Hz, H-6$_a$), 4.31 (dd, 1H, 5.9 and 11.6 Hz, H-6$_b$), 3.86-
3.76 (m, 4H, H-2, H-3, H-4, H-5), 3.75-3.56 (m, 2H, CH$_2$CH$_3$),
3.40 (s, 3H, 1-OMe), 1.21 (t, 3H, Hz, CH$_2$CH$_3$) ppm.

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EXAMPLE 6

Chaperone assays and the use thereof in testing compounds for inhibition of chaperone binding

In order to conclusively identify peptides or peptide mime-
5   tics that bind strongly to PapD, a good assay is required.
The maltose binding protein (MBP) has been engineered by the
inventors so that it is recognized by PapD. The commercially
available plasmid pMAL-P2 encoding MBP with a linker arm
encoding sequence fused to the 3' end of the gene was used.
10 MBP is secreted into the periplasmic space and is easily
purified by affinity chromatography using amylose resin. MBP
is eluted from the column with 20 mM maltose. PapD is not co-
eluted with MBP when co-expressed with MBP whereas fusing the
PapG COOH-terminus to MBP results in PapD binding to MBP to
15 form a complex that is co-eluted from the affinity column.
Using this strategy, it has been found that fusing the car-
boxy terminal 140 (MBP-G1'-140') and 134 (MBP-G134') amino
acids of PapG to MBP results in the formation of a strong
PapD-MBP complex which is purified by maltose affinity chro-
20 matography. The complex between the MBP fusion and PapD has
been shown to be stable in up to 3 M urea similar to the
stability of a PapD-PapG complex arguing that the COOH-ter-
minal 134 amino acids of PapG probably contains most of the
chaperone recognition motif. In addition, binding to the MBP
25 fusion protein has proven dependent on the universal chape-
rone anchoring residues, Arg-8 and Lys-112, as described
herein since mutations in these two residues abolished PapD
binding to the MBP fusion protein.

It is also interesting to test the ability of MBP-G fusions
30 to inhibit pilus assembly and thus bacterial attachment by
co-expressing the fusion proteins in cells producing P pili.
If PapD binds to the MBP-G fusion, it will be titrated away
from pilus subunits and thus decrease or abolish pilus forma-
tion. This technique will be one mechanism to validate the
35 concept that chaperone binding peptides can abolish the

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virulence of a pathogen by preventing the assembly of sur-
face-localized adhesins. Using this analysis it has been
found that in strains producing P pili, co-expression of the
MBP-G1'-140' and MBP-G134' fusion completely abolishes the
5   ability of the bacteria to bind red blood cells and cause
hemagglutionation. Further, it has recently been shown by the
inventors that after coexpression of the MBP-G1'-140' fusion
in HB101/pPAP5-bacteria, it was not possible to detect any
visual signs of pili on the surface of the bacteria by elec-
10   tron microscopy. This is evidence for the theory expressed in
this invention, that the prevention/inhibition of binding
between pilus subunits and molecular chaperones also prevents
the assembly of intact pili.

These results support the biological relevance of the crystal
15   structure described in Fig. 3, showing that other proteins
can be engineered to be recognized by PapD by fusing the
carboxy terminal recognition site onto that protein. The MBP-
G134' and MBP-G1'-140' fusions have been purified to estab-
lish in vitro assays to measure interactions with PapD. The
20   purified fusion proteins are coated on wells of microtiter
plates and the ability of PapD to bind to the fusion proteins
is tested in an ELISA procedure. This assay is critical since
it allows testing the ability of compounds to inhibit binding
of PapD to the domain on PapG recognized by the chaperone.
25   Furthermore, the purified fusion proteins can be used in the
Pharmacia BiaCore® assay to quantitate binding to PapD and
establish inhibition assays for compounds of the invention.
Carboxyl-terminal peptides have already been suggested to
correspond to part of the chaperone recognition motif. One
30   can thus investigate whether the carboxyl terminal peptides
are able to inhibit binding of PapD to the MBP-G1'-140'
fusion in the ELISA assay. The development of this high
through-put assay makes it possible to screen peptide
libraries, chemical libraries, natural compounds, and peptide
35   mimetics for their ability to inhibit chaperone binding. In
addition, this assay can be used to test whether the known
periplasmic chaperones utilize common recognition paradigms.

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In addition to testing compounds for their ability to inhibit binding to the MBP-G fusion, other assays have been established which measure PapD-peptide interactions. For example, an ELISA has been developed to measure the binding of PapD to
5   peptides coated on wells of microtiter plates. In this assay the octameric peptide G1'-8'WT was an equally efficient inhibitor as the 19-mer G1'-19'WT, revealing the octamer to be the optimal starting point for designing of modified compounds with increased affinity for the binding site of
10 PapD (cf. Example 7).

Also, as previously described, native PapD is able to bind to reduced, denatured PapG and restore the PapD-PapG complex in vitro in a reconstitution assay (Kuehn et al., 1991). This assay has been proposed to reflect the recognition function
15 of PapD in vivo and will be used to determine the ability of the compounds to inhibit PapD binding to PapG in vitro. For example, the carboxyl terminal PapG peptide has been shown to inhibit the binding of PapD to PapG in this assay, establishing that it occupies the pilus subunit binding site of PapD.
20 Another way to map PapD-peptide interactions is to test the ability of compounds to reduce the rate of well defined proteolytic cleavage events. For example, partial digestion with trypsin cleaves PapD in the F1-G1 loop at residue Lys-99 (See "T" site denoted in Fig. 3). The rate of tryptic clea-
25 vage of PapD was reduced by preincubation of PapD with PapG and PapK peptides (cf. Example 2). The observed protection of PapD by bound peptides may be due to a change of the local conformation of the F1-G1 loop, or due to physical contact of the loop by the peptide. These assays may thus be used as an
30 initial screening for the ability of new compounds to bind to PapD and interfere with its recognition function. Strong binding compounds that inhibit PapD binding to the MBP-G1'-140' fusion will be co-crystallized with PapD to provide the structural basis of the recognition surface used by PapD.
35 As new crystallographic data becomes available, the relevance of critical PapD-inhibitor or PapD-enhancer interactions will be tested by determining the effect of site-directed muta-

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tions on the ability of the chaperone to bind pilus subunits
and mediate pilus assembly. This important information will
lead to defining how chaperones recognize subunits and the
function of chaperone-subunit interactions.

5   A quantitative chaperone binding assay has also been deve-
loped: A modified PapG peptide has been synthesized, wherein
Ser-9' is substituted by a Cys. From the crystal structure of
PapD bound to the peptide, the side chain of Ser-9' was not
predicted to interact with PapD. Instead, this side chain was
10   oriented towards the solvent, but Ser-9' was adjacent to the
last amino acid of the interactive zipper between PapD and
the peptide. The environmentally sensitive fluorescent probe
5-IAF (5-iodoacetamidfluorescein) was then covalently coupled
to the peptide via the sulfhydryl group on the Cys residue
15   and the labelled peptide was purified; of course any other
suitable environmentally sensitive fluorescent probe may be
employed. It was found that the addition of PapD to the
peptide causes a marked decrease in the fluorescent intensity
and a shift in the emission maxima. This information was used
20   to calculate a binding constant for the PapD-peptide interac-
tion. The change in the fluorescence intensity at 514 nm
after the addition of increasing concentrations of PapD was
determined. By plotting the fluorescent intensities versus
PapD concentrations, a binding constant of 2.5×10^{-6} M was
25   calculated (The calculations were performed on the commer-
cially available computer program "Kaleidagraph").

It is thus possible to evaluate the binding constants of
other substances binding to PapD in a quantitative manner, as
the addition of a substance which binds competitively to PapD
30   will result in an increased fluorescence compared to a situa-
tion where less or no competitive substance is present in the
system.

A similar assay system will be developed using MBP-G1'-140'
instead of the PapG peptide as described herein. As this
35   fusion protein has been found to interact with two binding

sites in PapD, it will be possible to 1) quantitate the
binding affinity to the second binding site (on domain 2) and
2) screen for compounds which interact with either of the two
binding sites and quantitate the interaction. Of course,
5 labelling the site 2 peptide with a suitable environmentally
sensitive probe should allow a determination of the binding
constant using the same methodology as describe above.

The discovery that the DegP protease is greatly responsible
for the degradation of pilin subunits in the absence of a
10 chaperone renders the degP41 strain an interesting candidate
for an in vivo test system of the effects exerted on PapD by
compounds of the invention.

When administering a substance which prevents, inhibits or
enhances the binding between the chaperone and the pilus
15 subunits to a system containing the degP41 strain, the sub-
stance should, even in small amounts, be toxic to the
bacteria, as the DegP⁻ bacteria will be incapable of degra-
ding the accumulating pilus subunits. Note that a degP41
strain was used to identify a hitherto unknown binding site
20 on domain 2 of PapD, cf. example 10.

However, such an assay requires that the tested substance is
able to enter the periplasmic space before it can exert its
effect; this fact renders this type of assay less suitable as
a screening assay for lead compounds, as it will "ignore"
25 substances which have the desired effects on chaperone-sub-
unit interaction, but which are e.g. too hydrophillic to
enter the periplasmic space. On the other hand, the system
will be well-suited for assessing the clinical potential of
substances which have already proven successful in the in
30 vitro assays described herein.

Once the model PapD system is in place, the experiments
described above can of course be expanded to include the
other members of the PapD-like family of chaperones. In this
way it will be possible to establish the general requirements

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for chaperone recognition and the molecular basis of the chaperone recognition paradigm in gram negative bacteria.

Some specific assays for determination of interactions with molecular chaperones are described in detail in Example 10.

5 EXAMPLE 7

Design of peptides and peptide mimetics capable of binding to the binding site of PapD and other pilus assembling chaperones

To provide further information on chaperone-subunit interac-
10 tions, it is contemplated to synthesize peptides overlapping the entire PapG protein and other pilus subunits. In addition, probing peptide binding to available chaperones using both a chemical peptide library and a phage display library should be performed. The two libraries are complementary
15 since the peptides are presented in different environments which could importantly influence binding to the chaperones. In practice the chemical library is limited to all possible hexapeptides and to hepta- and octapeptides if some of the natural amino acids are excluded or if one or two residues
20 are kept invariant. This library can be evaluated by direct binding of the chaperone to the resin beads and sequencing of interesting peptides. The phage display library contains approximately 10^{10} peptides in the PIII protein of the so-called "fusion phage" that retains phage function and dis-
25 plays the foreign peptides on the surface. The ability of peptide-containing phages to bind PapD coated in wells of microtiter plates can be detected in an ELISA using the techniques described in Example 6. Positive binding phages will be amplified, purified and retested for their ability to
30 bind PapD. The sequence of the peptide insert of positively binding phages will be determined and the corresponding peptides will be synthesized and tested for their ability to inhibit biding of PapD to the MBP-G fusion as described in Example 6.

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The results from the studies outlined above will be of the utmost importance for design and evaluation of ligands acting as "chaperone inhibitors". In spite of this, the crystal structure of the PapD-PapG 19-mer peptide complex provides

5 sufficient insight into chaperone-ligand interactions to initiate systematic studies of small peptides and peptide mimetics as chaperone inhibitors already at the present stage. The high inhibitory power of the PapG 8-mer peptide to PapD (equal to the PapG 19-mer peptide, cf. Example 6)

10 reveals the feasibility of such studies and the conserved features of the proposed binding site on the chaperones indicate that such inhibitors could well have broad specificity.

The specificity in recognition of the PapG 19-mer by PapD has

15 been suggested to be provided by anchoring of the peptide's carboxyl terminus to the PapD cleft residues Arg-8 and Lys-112 and subsequent "zippering" interactions between alternating hydrophobic residues in the peptide and complementary hydrophobic residues in PapD. The following experiments

20 support this hypothesis. Firstly, mutations in the anchor residues Arg-8 and Lys-112 have abolished subunit binding in vivo. Secondly, deletion of the C-terminal residue in the PapG 19-mer leads to a substantially decreased binding to PapD as the hydrophobic "zippering" interactions between

25 peptide and PapD were then placed off register when the C-terminus of the deletion peptide was anchored to Arg-8 and Lys-112. The "zippering" hypothesis for specificity in binding between PapD and peptides (or pilus subunits) has been preliminary investigated by performing the following experi-

30 ments:

A length series of peptides form the C-terminus of PapG consisting of G1'-5', G1'-6', G1'-7', G1'-8', G1'-11', G1'-16' and G1'-19' was prepared. A replacement series and a deletion series of G1'-8' was also synthesized. In the repla-

35 cement series Pro-1', Phe-2', Leu-4', Val-5', Met-6' and Met-8' was replaced by Ser, whereas Ser-3' and Thr-7' was

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replaced by Ala (i.e. hydrophobic amino acids was replaced by
the polar Ser and hydrophillic amino acids was replaced by
Ala). In the deletion series one residue at a time in G1'-8'
was deleted with simultaneous addition of a serine (which is
5   found in position 9' in native PapG) to maintain the peptide
length at 8 amino acids. The peptides were all synthesized by
the Fmoc solid phase strategy and were purified by reversed
phase HPLC.

The ability of the peptides to inhibit the binding of PapD to
10 the MBP-G1'-140' fusion protein was then investigated using
the following ELISA test:

Stock solutions of MBP-G1'-140' proteins in PBS were diluted
to 0.1 μM with PBS. The wells of microtiter plates were
coated with 50 μl of the protein solutions overnight at 4°C.
15 The wells were washed with PBS, and blocked with 200 μl of 3%
bovine serum albumin (BSA) in PBS for 2 hours at 25°C. The
plates were washed vigorously three times with PBS and incu-
bated with 50 μl of 1-5 μM PapD proteins in 3% BSA-PBS for 45
minutes at 25°C. The PapD was preincubated with each of the
20 peptides at a 1:25 ratio for 30 min before being added to the
wells. After three washings with PBS, the wells were incu-
bated with a 1:500 dilution of rabbit anti-PapD antiserum in
3% BSA-PBS for 45 minutes at 25°C. After three washings with
PBS, the wells were incubated with a 1:1000 dilution of goat
25 antiserum to rabbit IgG coupled to alkaline phosphatase in 3%
BSA-PBS for 45 minutes at 25 °C. After three washings with
PBS and three washings with developing buffer (10 mM diethan-
olamine, 0.5 mM $MgCl_2$), 50 μl filtered 1 mg/ml p-nitrophenyl
phosphate in developing buffer was added, the reaction was
30 incubated for 1 hour or longer if necessary in the dark at
25°C, and the absorbance at 405 nm was read.

The inhibitory powers of the peptides in the three series are
presented in Figs. 17-19 and the number of experiments per-
formed with each series is given in the Figs. Vertical lines
35 for each peptide in the figures are 95% confidence intervals

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obtained after a statistical analysis of the experimental
data. As revealed by the evaluation of the length series, the
peptides G1'-8', G1'-11' and G1'-16' are significantly more
potent than the shorter G1'-6' and G1'-7' (Fig. 17). This
5   observation fits very well with the crystal structure of PapD
complexed with G1'-19' which shows that the C-terminal 8
residues in G1'-19' are hydrogen bonded to PapD. The shorter
peptides G1'-6' and G1'-7' are unable to fulfil this hydrogen
bonding pattern and are therefore less active inhibitory
10   peptides (Fig. 18). The replacement series reveals that
residues 4', 5' and 6' in G1'-8' form important contacts with
PapD, since their replacement results in less active inhibi-
tory peptides (Fig. 18). The deletion series again indicated
an important role for residues 4', 5' and 6' in G1'-8' for
15   the complex formation with PapD (Fig. 19). However, the
results obtained with the deletion series did not support the
"zippering" hypothesis according to which the members of the
deletion series would show an increasing inhibitory potency
as the deletion is moved form the C-terminus towards the N-
20   terminus of G1'-8'.

As appears from these preliminary results, the results
obtained in the assay exhibit large deviations from the mean
in each experiment. As discussed herein, one reason for this
might be the slow kinetics of binding between PapD and cor-
25   rectly folded pilus subunit proteins and analogues of such
correctly folded subunit proteins. It is therefore contem-
plated to modify the assay by introducing denaturing influ-
ences powerful enough to at least partially unfold the pilus
subunit (analogues). It is expected that this will reduce the
30   deviations in the assay results. Another reason for the large
deviations might be binding of the peptides to BSA used in
the ELISA. Therefore, the replacement of BSA with other
macromolecules will be investigated.

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In case the "zipper mechanism" for peptide binding to PapD
can be confirmed, peptide chaperone binding can be optimized
using a limited synthetic peptide library in which Pro-1',
Phe-2', Leu-4', Met-6' and Met-8' of the PapG 8-mer are
5   replaced by the hydrophobic amino acids Val, Leu, Ile, Met,
Phe, Trp, Tyr and His. Non-natural amino acids such as D-
amino acids and N-methylated amino acids, as well as amino
acids containing aliphatic and various aromatic side chains,
will also be incorporated in the chemical library. Optimal
10   amino acid combinations for these five positions will be used
in the synthesis of the chaperone inhibitors described below
in this Example. If the "zipper mechanism" is not confirmed,
the outlined approaches will be applied to residues shown to
be important for binding to PapD.

15   Chaperone inhibitors that form covalent bonds to the chapero-
ne after docking into its active site will be developed. The
crystal structure of the PapD-peptide complex shows that the
C-terminal carboxyl group of the peptide is hydrogen bonded
to Lys-112 and Arg-8 in the chaperone and that the side chain
20   of Val-5' in the peptide is close to the side chain amino
group of another Lys in PapD. Introduction of reactive groups
such as alkyl halides, aldehydes, acid halides and active
esters in these positions of the optimized 8-mer peptide will
lead to the formation of covalent bonds to the lysines in
25   PapD, and the peptide derivatives thus constitute high affin-
ity inhibitors. The inhibitors are based on nonamer or
shorter peptides in which residue 5 (from the C-terminus)
and/or the C-terminal carboxyl group have been modified as
indicated:

Replacements for residue 5 from the C-terminus:

$n = 0-5$

$R = H, Me, Et, C_3H_7, C_4H_9, C_6H_5$

$X = H, F, Cl, Br,$

$R_a = F, Cl, Br$

$R_a = R_c = H, R_b = NO_2$
$R_a = R_b = NO_2, R_c = H$
$R_a = R_b = R_c = NO_2$
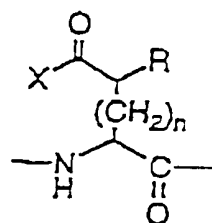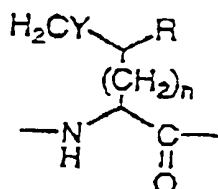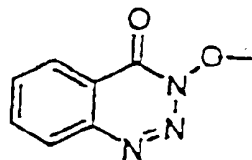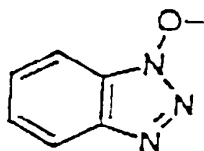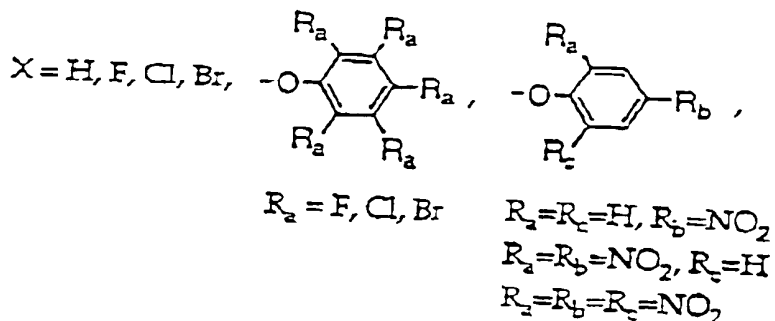
n and R as above

$Y = Cl, Br, I, OSO_2Me, OSO_2CF_3, OSO_2C_6H_4Me$

Replacements for the C-terminal COOH-group:

-COX, X as above

-CH$_2$Y, Y as above

Such interactions between aldehydes and lysine side chains have given potent drug candidates for sickle-cell anaemia.

In the *PapD*-peptide crystal, the peptide forms an extension of a β-sheet in the chaperone. Restrictions that give the peptide a β-sheet like conformation will therefore result in a favourable change in the entropy of binding. Conformation-

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ally restricted peptides constituting miniature cyclic β-
sheets or peptides having the side chains of amino acids
separated by one amino acid covalently linked to each other
will be prepared based on the optimized 8-mer peptide. Pepti-
5   des with covalently linked side chains will have the three
consecutive amino acids replaced by the fragments shown
below:
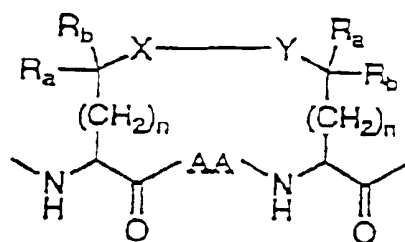
$X,Y = S,S; S,CH_2; CH_2,S; O,CH_2;$
$CH_2,O; CH_2,CH_2$
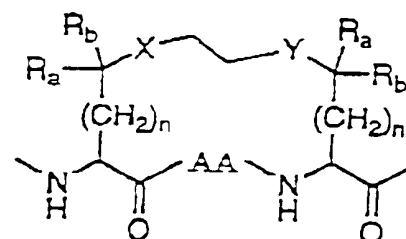
$X,Y = S,S; S,CH_2; CH_2,S; O,O;$
$O,CH_2; CH_2,O; CH_2,CH_2$

AA = any natural or nonnatural amino acid
R_a,R_b = all possible combinations of H, Me, Et, C_3H_7, C_4H_9, C_6H_5


AA = any natural or nonnatural amino acid
R_a,R_b = all possible combinations of H, Me, Et, C_3H_7, C_4H_9, C_6H_5

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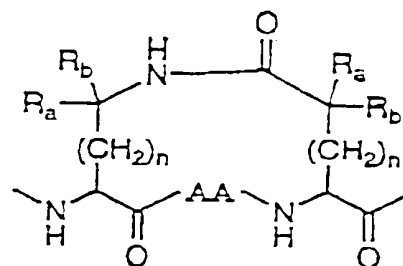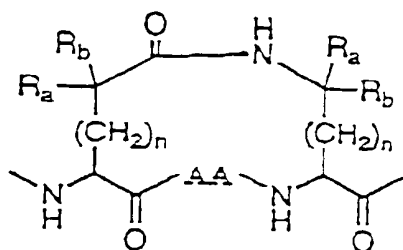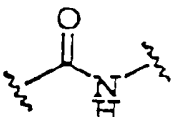
Interesting inhibitors will be co-crystallized with chaperones, and the complexes will also be investigated by NMR spectroscopy.

5 Peptides and peptide mimetics used as drugs can be rapidly metabolized by proteolytic enzymes that cleave the peptide bonds. Chymotrypsin selectively cleaves the peptide bond on the carboxyl side of amino acids with aromatic and large hydrophobic side chains. In the *PapG* 8-mer, the Met-8'-Thr-7', Met-6'-Val-5' and Phe-2'-Pro-1' amide bonds are
10 therefore especially sensitive to proteolysis and should be replaced by metabolically stable peptide isosters. Examples of such peptide isosters are given below:

Replacements for the $\overset{O}{\overset{\|}{C}}-\overset{H}{\underset{|}{N}}$ fragment:

-CH₂CH₂-
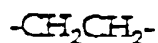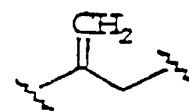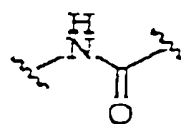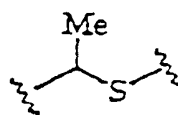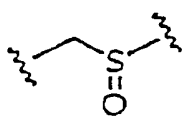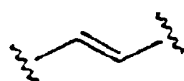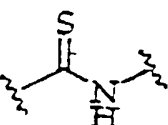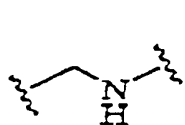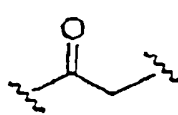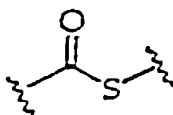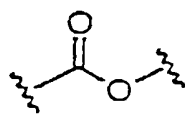
EXAMPLE 8

The role of periplasmic chaperones in the infectivity of bacteria which adhere by the means of pili

The specific role of chaperone-assisted pilus assembly in
5 virulence can be determined by comparing the adherence and
pathogenicity of piliated wild type and isogenic mutants that
are non-piliated due to site-directed mutations in the active
site of the chaperone. Mutations in residues such as Arg-8
and Lys-112 that constitute the subunit binding site of *PapD*
10 can be recombined into the bacterial chromosome of the clini-

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cal isolate strain DS17 using the same method that has been
successful to introduce point mutations into the papG gene in
the chromosome of the same strain. *E. coli* strain DS17 spread
epidemically within a neonatal ward, causing several cases of
5 pyelonephritis. In addition, the strain has been found to
cause acute kidney infections in a cynomolgus monkey
pyelonephritis model. DS17 contains one pap gene cluster with
a papG gene expressing a typical papG adhesin. It also
expresses typical type 1 pili containing the mannose-binding
10 adhesin, FimH. Type 1 pili have been suggested to be impor-
tant virulence determinants in cystitis. Therefore, site-
directed mutants in the subunit binding site of the *FimC*
chaperone should also be generated to test this concept.

It is contemplated that a compound that binds the chaperone
15 active site would block pilus assembly and thus prevent
attachment. If this concept is valid, then well-defined
chaperone mutants such as in Arg-8 and Lys-112 should abolish
receptor binding, too. The receptor binding activity of
various chaperone mutants should be measured in the receptor
20 binding ELISA assays described in examples 2 and 6. To
measure type 1 pilus-mediated binding, mannose will be linked
to the wells of the microtiter plates. Adherence of various
FimC and *PapD* chaperone mutants to the immobilized receptors
will be quantitated using antibodies to *E. coli* DS17 in an
25 ELISA experiment. Furthermore, the ability of small *PapD*-
binding peptides to block pilus assembly in DS17 and, thus,
to abolish receptor binding will be tested. Strain DS17 will
be grown in the presence of short *PapD*-binding or non-binding
peptides and then tested for their ability to bind receptor
30 in the ELISA experiment. These experiments will make impor-
tant strides at validation the concept that anti-chaperone
inhibitors would prevent bacterial attachment *in vitro*.

Then, the role of chaperone-assembled adhesins in causing
diseases will be established. The causality between *E. coli*
35 expressing P pili and pyelonephritis has until recently been
based solely on epidemiological data. It has been shown that

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the P piliated strain DS17 causes pyelonephritis to occur in
the normal urinary tract of cynomolgus monkeys. A mutation in
the papG gene of strain DS17 was generated by allelic re-
placement to create the derivative strain DS17-8 to test the
5 requirement of the PapG adhesin in causing the disease. DS17-
8 contains one base pair deletion after codon 37 in papG
resulting in the expression of P-pili lacking the PapG tip
adhesin. This mutant was unable to bind the globoside
receptor in vitro and was unable to bind to kidney tissue
10 from humans or cynomolgus monkeys in our in situ attachment
model. The virulence between DS17 and its papG mutant DS17-8
in the cynomolgus monkey was compared. To study the role of
the PapG adhesin in pyelonephritis, five monkeys were
infected with E. coli strain DS17 whereas six monkeys
15 received mutant strain DS17-8 via a cytoscopically inserted
ureteral catheter, and it was shown that there was a signifi-
cant difference between the two groups. The monkeys receiving
wild type strain DS17 has a mean bacteruria of 21 days com-
pared to 6.8 days for those monkeys receiving the mutant
20 strain DS17-8. Renal clearance was also significantly lower
for the wild type strain and renal function was significantly
reduced in the monkeys receiving the wild type strain but not
in those monkeys receiving the isogenic papG mutant strain.
Pathologic evaluation of the infected kidney confirmed the
25 functional studies showing significantly less inflammation
and pathologic changes in the mutant group as compared to
those receiving the wild type.

Therefore, it is concluded that the Galα1-4Gal binding PapG
adhesin at the tip of P pili is required for pyelonephritis
30 to occur in the normal urinary tract of primates. Up to now,
no such direct demonstration for the role of a pilus associ-
ated adhesin in a specific bacterial infection has been made.

Interestingly, in the experiments described above, no evi-
dence was found that the PapG adhesin was required for colo-
35 nization of the lower urinary tract or for the development of
acute cystitis. Both strains colonized the vagina, persisted

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in the intestine and caused bladder infection. Thus, although
PapG was a critical virulence determinant in causing
pyelonephritis, it did not appear to be crucial in cystitis.
However, this did not exclude the possibility that other
5   components of the P pilus or that another type of pilus, such
as type 1, was required for cystitis.

These hypotheses will be investigated by testing the viru-
lence of isogenic DS17 mutant strains containing site
directed mutations in Arg-8 of PapD or FimC. The mutants will
10   presumably be defective in their ability to assemble P and
type 1 pili. If the papD mutant is non-virulent in the monkey
cystitis model, it may indicate that some component of the P-
pilus other than PapG is essential for E. coli to generate
bladder infection. For example, the major component of the
15   tip fibrillum, PapE, has been shown to bind fibronectin and
fibronectin could be an important factor in cystitis. Simi-
larly, it the fimC mutant is non-virulent, it would confirm
the role of the mannose-binding type 1 pili in causing cysti-
tis. The ability of these point mutations to abolish the
20   virulence of DS17 in causing either pyelonephritis or cysti-
tis would validate the therapeutic potentials for a chaperone
inhibitor.

EXAMPLE 9

Identification of the motif of binding between PapD and
25   K1'-19'WT

In order to confirm the generality of the binding mode for
C-terminal peptides of pilin subunits to PapD as observed in
the PapD-G1'-19'WT crystal structure (see Example 1) a second
PapD-peptide complex was investigated by X-ray crystallo-
30   graphy. Since tight binding had been observed for the peptide
derived from the wild-type C-terminal 19 amino-acids of PapK
(K1'-19'WT, SEQ ID NO: 18, and numbered here from the C-ter-
minal Arg-1' to the N-terminal Lys-19') it was decided to use
this as the peptide for the second PapD complex.

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Obtaining material; protein & peptide.

PapD was prepared as previously described (Holmgren et al., 1988) and obtained from Dr. Scott Hultgren, Dept of Molecular Microbiology, Washington University School of Medicine, St.
5   Louis USA. The peptide K1'-19'WT was prepared by Fmoc solid phase synthesis, purified by reversed phase HPLC and obtained from Dr. Jan Kihlberg, Dept. of Chemistry, University of Lund, Lund, Sweden.

Crystallisation of PapD-peptide complex.

10   After a number of different experimental conditions had been explored around those previously used to obtain *PapD*-G1'-19'WT crystals the best crystals of the *PapD*-K1'-19'WT complex were grown by vapour diffusion against 20% PEG8000, 0.1 M MES pH 6.5. The crystallisation drop
15   contained equal volumes of reservoir and protein solution. The protein solution (15 mg/ml) contained a 1:1 molar ratio of *PapD* to peptide in 20 mM MES pH 6.5 with 1.0% β-octyl glucoside (β-OG).

These crystals were mounted inside sealed quartz-glass capil-
20   lary tubes and initially characterised by examining them on a X-ray precession camera. From standard analysis of such images it was determined that the above-mentioned crystals have a orthorhombic space group, C2221 (thus differing from the *PapD*-G1'-19'WT crystals which were C2), with cell dimen-
25   sions a=57.1 Å, b=153.2 Å, c=135.4 Å and $\alpha=\beta=\gamma=90°$, 2 mole-cules in the asymmetric unit and diffract to 2.7 Å resolution on a lab X-ray source with rotating anode and Cu K_α target.

Collection & processing of experimental data.

The intensity data for the *PapD*-K-peptide crystals were
30   collected on a R-AXIS II area-detector system (from R-AXIS) at Symbicom AB., Uppsala. All data were obtained from a single crystal and processed initially with the DENZO soft-

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ware package (Otwinsky, 1993). Merging and scaling of the
data, however, was carried out using ROTAVATA and AGROVATA
from the CCP4 package (CCP4, 1979). The final data set con-
tained 15,989 independent reflections with an Rsym of 8.7%
5   for data between 20.0 and 2.7 Å resolution.

Solution of three dimensional structure.

The structure of the complex was solved by the standard
method of molecular replacement using the program XPLOR
(Brunger, 1992). The search model used was the refined 2.0 Å
10   resolution structure of *PapD* (Holmgren and Bränden, 1989.
Using 8.0 to 4.0 Å resolution data the self-rotation function
again gave a clear non-crystallographic two-fold axis. The
top peaks in the translation functions also gave the correct
solutions. After the translation functions the R-factor was
15   36.7% for 8.0 to 4.0 Å resolution data. Subsequent rigid body
refinement in which all 4 domains of the 2 *PapD* molecules in
the asymmetric unit were allowed to refine independently
resulted in an R-factor of 33.6% for the same data.

Examination of an |Fo|-|Fc| electron density map at this
20   stage using the graphics program O (Jones and Kjeldgaard,
1994) showed clear density corresponding to the K-peptide in
the *PapD* cleft and running along the surface of the protein
in an analogous fashion to that found for the G-peptide. The
orientation of the peptide was easily determined from the
25   electron density, but initially only the final 12 C-terminal
amino-acids of the peptide could be modelled into density.

Refinement and analysis of structure.

Simulated annealing refinement with XPLOR (Brunger, 1992) was
initiated at this stage. Several additional cycles of model
30   building and refinement were carried out with a further 2
peptide amino-acids being added to the N terminal end of the
peptide to yield an R-factor for the current model of 19.2%
for 8.0 to 2.7 Å resolution data. The model at the present

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stage of refinement (which contains no water molecules and
does not include the first 5 N-terminal amino-acids of the
peptide) has root-mean square (rms) deviations from ideal
geometry of 0.019 for bonds lengths and 3.8° for bond angles.

5   Despite the two PapD-peptide complexes being solved in dif-
ferent space groups, their overall structures are found to be
highly similar with the peptide interacting with PapD in a
manner essentially identical to that previously seen for the
PapD-G1'-19'WT structure. Thus the K1'-19'WT peptide is again
10 seen to bind in an extended conformation with the C-terminal
Arg-1' anchored within the inter-domain cleft and subunit
binding site. Hydrogen bonds are also formed between the
peptide carboxy terminus and two invariant positively charged
residues of PapD, Arg-8 and Lys-112. The K1'-19'WT peptide
15 then run along the surface of the N-terminal domain, forming
a parallel β-strand interaction with strand G1. In this way
between 9 mainchain hydrogen bonds are formed between resi-
dues 10' to 2' of the peptide and Val-102 to Lys-110 of PapD
and thus extending the β-sheet of PapD out into the peptide.

20 In addition, a dimer association is observed between the two
PapD-peptide complexes within the unit cell similar to that
seen in the PapD-G1'-19'WT crystal structure. Again the
β-sheet is extended as a result of a non-crystallographic
2-fold symmetry which places a second PapD-peptide complex
25 adjacent to the first such that the two bound peptide chains
interact as anti-parallel β-strands with a mixed β-sheet
again being created between the two complexes involving a
total of 10 β-strands. The only major difference between the
two PapD-peptide complexes is the fact that in the
30 PapD-K1'-19'WT structure the peptides of the non-
crystallographically related complexes are positioned two
residues closer to the COOH-terminus of its partner. Thus
while eight hydrogen bonds are formed between the peptides in
the PapD-G1'-19'WT complex a total of 10 are observed in the
35 PapD-K1'-19'WT.

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Apart from the C-terminal residues Tyr-2' and Arg-1' there are again relatively few contacts between the sidechains of the peptide and PapD. The major interactions are provided by the mainchain hydrogen bonds to the G1 strand. There are,

5   however, a number of hydrophobic interactions within the β-sheet, in particular between the peptide's Tyr-6' with Ile-105 and Leu-107 of strand G1.

EXAMPLE 10

Identification of a second binding site in PapD and develop-
10 ment of new assays

Introduction

A chaperone binding assay was developed to delineate PapD-PapG interactions using MBP/G fusions, containing increasing lengths of the COOH-terminus of PapG. The ability of PapD to
15 bind to PapG truncate proteins missing increasing lengths of the COOH terminus of PapG was also examined.

Experimental procedures

Bacterial strains

The E. coli strain HB101 (Maniatis et al., 1982) was used as
20 a host strain in the studies involving the MBP/G fusion proteins. Strain DH5α was used as the host to construct MBP/G fusion proteins (Hanahan, 1983) and PapG truncates. KS474 (degP::kan) (Strauch et al., 1989) was kindly provided by J. Beckwith and used for expression of the PapG truncate pro-
25 teins.

Plasmid construction

The E. coli expression plasmid pMAL-p2 (new England Biolabs, Beverly, MA, USA) was used for the construction of pMAL-p4, pMAL/G1'-19', pMAL/G1'-81' and pMAL/G1'-140' using procedures

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essentially from Maniatis (Maniatis et al. 1982). The *malE*
gene encoded by pMAL-p2 has a *lacZα* sequence fused to the 3'
end. Plasmid pMAL-p2 was digested with *SalI* and filled in
with Klenow (Maniatis et al., 1982). The fragment was re-
5   ligated and the resulting plasmid called pMAL-p4 had a stop
codon between the *malE* and *lacZα* sequences. Plasmid pMAL-p4
was digested with *HindIII* and filled in with Klenow and the
resulting fragment was ligated with a *ClaI* linker,
5'CCATCGATGG3' (New England Biolabs, Beverly, MA, USA) to
10   produce plasmid pMAL-p5. Primer 23969
(5'CCCCCCTGCAGATCAGATTAAGCAGCTACCTGC3', SEQ ID NO: 23) and
primer 23557 (5'CCCCTGCAGTAAAAATATCTCTGCTCAGAAATAC3', SEQ ID
NO: 24) and primer 23559 (5'CCATCGATGAACAGCCAGTCAGATAATC3',
SEQ ID NO: 25) were used for Polymerase Chain Reactions (PCR)
15   utilizing pPAP5 (Hull et al., 1981; Lindberg et al., 1984) as
the template. The DNA fragments encoding the region of amino
acid residues 1'-81' and 1'-140' of the *PapG* were obtained
from the PCR reactions using primers 23557 and 23559; and
primers 23969 and 23559, respectively. The amplified DNA
20   fragments were purified and restricted with *PstI* and *ClaI* and
ligated into *PstI* and *ClaI* restricted pMAL-p5 fragment to
construct pMAL/G1'-81' and pMAL/G1'-140', respectively. To
create pMAL/G1'-19', two nucleotide oligomers
(5'GGAAAGAGAAACCCGGGGAGCTATCTGGTTCTATGACTATGGTTCTGAGTTTCCCCT
25   GAT3', SEQ ID NO: 26 and
5'CGATCAGGGGAAACTCAGAACCATAGTCATAGAACCAGATAGCCCCGGGTTTTCTCTTT
CCTGCA3', SEQ ID NO: 27) were chemically synthesized and
annealed. The annealed DNA fragment containing the sequence
of the carboxyl terminal 1'-19' amino acid residues of *PapG*
30   was ligated to *PstI-ClaI* pMAL-p5 fragment to produce
pMAL/G1'-19'. All the constructs used in this study were
confirmed by DNA sequencing analysis using Sequenase Version
2.0 according to the manufacturer's directions (USB, Cleve-
land, OH, USA). pHJ14 (*PapG3*) was created by digestion of
35   pHJ8 (wt *PapG* in P_{tac} promoter plasmid, pMMB66) with *BamHI*
and *BglII*, removing approximately 500 nucleotides, purifica-
tion of the large fragment and religation. pHJ23 (*PapG 2*) was
created by first cloning *papG* into pUC19 at *EcoRI* and *BamHI*,

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digesting with *HincII* to remove the last approximately 300
nucleotides and religating. The *EcoRI-HincII* fragment was
then cloned into pMMB66 to create pHJ23.

Induction and partial purification of MBP/G fusions

5   Strains carrying MBP or MBP/G fusion gene plasmids were
induced in LB broth culture with 1 mM isopropylthiogalacto-
side (IPTG). Periplasmic extracts were prepared as described
(Slonim et al., 1992). One tenth volume of 10X phosphate-
buffered saline (PBS, 120 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4
10   and 2 mM KH_2PO_4, pH 7.4) was added to the periplasmic
extract. Amylose resin was then added to the periplasmic
extracts at a 1:5 ratio. The mixture was rocked at 4°C over-
night and the beads were subsequently washed 5 times with
PBS. MBP or MBP/G fusion proteins were eluted with 20 mM
15   maltose in PBS by rocking at 4°C for an hour. Protein concen-
trations were determined using the Bio-Rad DC protein assay
kit (Bio-Rad Laboratories, Hercules, CA, USA). The full
length fusion protein concentration was further quantitated
by the Coomassie stained SDS-PAGE gel with known concentra-
20   tions of bovine albumin (BSA) as the standards followed by
densitometry scanning.

*Characterization of interactions between PapD and MBP/G
fusion proteins*

The MBP or MBP/G fusion preparations from HB101/pLS101/pMAL-
25   p4, HB101/pLS101/pMAL/G1'-19', HB101/pLS101/pMAL/G1'-81' and
BH101/pLS101/pMAL/G1'-140' were applied to isoelectric focu-
sing (IEF) pI 3-9 gels (Pharmacia Phast System, Pharmacia,
Sweden) or 12.5% SDS-PAGE followed by silver staining, Coo-
massie blue staining or immunoblotting with anti-*PapD*
30   antiserum as described (Kuehn, et al., 1991).

The stability of the *PapD*-MBP/G1'-140' complex in urea was
determined by incubating 1 μg partially purified MBP/G1'-140'
preparation from HB101/pLS101/pMAL-G140 in 0-5 M urea for 5

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minutes at 25°C (Kuehn et al., 1991). The proteins were analyzed by IEF (pI 3-9) with silver staining. The PapD-MBP/G1'-140' complex on the IEF gel was quantified by densitometry (Digital Imaging System, Is-1000).

5   The interaction between PapD and MBP/G fusion in vitro was assayed by incubating purified PapD (1 µg) and amylose bead affinity purified MBP/G fusion protein (1 µg) in 4 µl PBS for 30 minutes and then applied to IEF (pI 3-9), followed by silver staining. Another in vitro assay that is more quanti-
10 tative is the ELISA described below.

Characterization of the interaction between PapD and PapG truncates

PapD and the PapG-truncates were co-expressed in KS474, the truncates were induced at OD_{600} 0.6 with 0.5 mM IPTG and PapD
15 under the control of the arabinose promoter was induced with 0.2% arabinose. After one hour induction, periplasmic extracts were prepared and rocked with Gal α(1-4) Gal beads overnight at 4°C and eluted with 15 µg/ml Gal α(1-4) Gal-TMSET solution as described previously (Hultgren et al.,
20 1989; Kuehn et al., 1991). The eluates were analyzed by acidic native gel eletrophoresis (Striker et al., 1994) followed by western blotting using anti-PapD and anti-PapG antiserum.

Hemagglutination titers

25 HB101/pFJ3 harbouring various MBP/G fusion plasmids was passaged on Trypticase Soy Agar (TSA, Becton Dickinson, Cockeysville, MD, USA) three times to induce P-pili expression. At the last passage, the expression of MBP/G fusions was induced on TSA containing 100 µM IPTG. The cells from
30 different strains were then collected and the HA titers were determined as described (Jacob-Dubuisson et al., 1993b).

Pili preparation and quantitation

The cells obtained from TSA plates were collected. Pili were prepared from the same amounts of cells using the procedure described previously (Jacob-Dubuisson, et al., 1993b). These
5   pili preparations were boiled in Lammeli sample buffer with 4 M urea, then analyzed on Coomassie blue stained SDS polyacrylamide gels. The relative amount of piliation was quantified by densitometric scanning of the PapA bands.

ELISA assay

10 Stock solutions of MBP/G fusion proteins in PBS were diluted to 40 pmol/50 μl with PBS. The solutions of the 40 pmol/50 μl MBP/G fusion proteins were serially diluted in PBS and 50 μl of each solution were used to coat the wells of a 96-well Nunc Immunoplate (Inter Med, DK-4000 Roskilde, Denmark)
15 overnight at 4°C. The wells were then washed with PBS and blocked with 200 μl of 3% BSA in PBS for 2 hours at 25°C. The plates were washed vigorously three times with PBS and incubated with 50 μl of *PapD* diluted at 50 pmol *PapD* in 50 μl 3% BSA-PBS for 45 minutes at 25°C. After three washings with
20 PBS, the wells were incubated with a 1:500 dilution of rabbit anti-*PapD* antiserum in 3% BSA-PBS for 45 minutes at 25°C. After three washings with PBS, the wells were incubated with a 1:1000 dilution of goat antiserum to rabbit IgG coupled to alkaline phosphatase in 3% BSA-PBS for 45 minutes at 25°C.
25 After three washings with PBS and three washings with developing buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$), 50 μl of filtered 1 mg/ml p-nitrophenyl phosphate (Sigma, St. Louis, MO, USA) in developing buffer was added. The reaction was incubated for 1 hour in the dark at 25°C and the absorbance
30 at 405 nm was read.

For assaying the second site peptides, all the peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) to a final concentration 2 mM. The stock solutions were diluted to a proper concentration in PBS and coated overnight

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on microtiter wells at 4°C. All the peptide solutions were
adjusted to the same DMSO concentration. The subsequent steps
followed the procedure described above.

Results

5   *MBP/G fusions*

To identify determinants on *PapG* essential for *PapD* interac-
tion we constructed three in frame fusions between the *MalE*
gene, encoding maltose binding protein, and sequences enco-
ding the COOH-terminal residues 1'-19', 1'-81' and 1'-140' of
10   *PapG* (Fig. 20). The resulting chimeric proteins were called
MBP/G1'-19', MBP/G1'-81' and MBP/G1'-140', respectively, to
indicate the regions of *PapG* present in the MBP/G fusions.
MBP/G1'-19' was created since *PapD* was recently shown to bind
a peptide consisting of the C-terminal 19 amino acids of *PapD*
15   *in vitro* (Kuehn et al., 1993). MBP/G1'-81' and MBP/G1'-140'
were created to test the requirement of the disulfide bridge
(C196-C228 in *PapG*) for recognition by *PapD*. A similarly
located disulfide bond is found in virtually all pilus sub-
units (Simons et al., 1990). MBP/G1'-81' lacks the two
20   cysteine residues while MBP/G1'-140' contains the disulfide
bond (Fig. 20).

PapD-MBP/G interactions in vivo

The ability of *PapD* to bind to the MBP/G fusions was investi-
gated using amylose affinity chromatography (Kellermann and
25   Ferenci, 1982). *PapD* was co-expressed from plasmid pLS101
(Slonim et al., 1992) with each of the MBP/G fusions. Peri-
plasmic extracts from each strain containing the MBP/G pro-
teins and *PapD* were subjected to amylose affinity chromato-
graphy and the eluates analyzed by SDS-PAGE (Fig. 21A) and by
30   western blotting with anti-*PapD* antisera (Fig. 21B). In this
assay, co-elution of *PapD* signified the ability of *PapD* to
interact with the MBP/G protein. The western blot revealed
that *PapD* co-eluted with all three fusion proteins (Fig. 21B,

lane 2, 3 and 4) but not with the MBP control (Fig. 21A and 21B, lane 1). However, PapD interacted much stronger with the MBP/G1'-140' fusion as can be seen in the western blot (Fig. 21B, lane 4) as well as in the coomassie blue stained gel of
5   the eluates (Fig. 21A, lane 4). Thus, PapD interacted strongly with the MBP/G1'-140' protein and only weakly with the MBP/G1'-19' and MBP/G1'-81' proteins.

Analysis of the eluates on silver stained isoelectric focusing (IEF) gels revealed that the PapD-MBP/G1'-140' complex
10 migrated at an isoelectric point (pI) of 5.2 (Fig. 21C, lane 4) which was intermediate between the pIs of MBP/G1'-140' (~4.4) and PapD (~9.1). This band was confirmed to contain both PapD and MBP/G1'-140' by excising the unstained band, applying the material to SDS-PAGE and analyzing it by wes-
15 tern-blotting using anti-PapD and anti-MBP antisera (Fig. 21D). It was not possible to detect stable complexes in the eluates of MBP, MBP/G1'-19' or MBP/G1'-81' when co-expressed with PapD (Fig. 21C, lane 1, 2 and 3).

The stability of the PapD-MBP/G1'-140' complex was measured
20 as a function of urea concentration in the presence of 15 mM DTT. Both the PapD-PapG and PapD-MBP/G1'-140' complexes behaved similarly under these conditions and were dissociated after incubation in 2M urea (data not shown). By these criteria, the PapD-MBP/G1'-140' complex was as stable as the
25 PapD-PapG complex.

Expression of MBP/G proteins inhibit pilus formation

PapD is essential for P-pilus assembly (Hultgren et al., 1991). A decrease in the concentration of PapD in the periplasm has been shown to cause a concomitant decrease in
30 piliation (Slonim et al., 1992). The ability of the MBP/G fusions to block pilus formation by inhibiting chaperone-subunit complex formation when co-expressed in trans with the pap operon was tested. Plasmids pMAL-p4, pMAL/G1'-19', pMAL/G1'-81' and pMAL/G1'-140' were transformed into the

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strain HB101/pFJ3 which contains the pap operon under the
control of its own promoter in the vector pACYC184 (Jacob-
Dubuisson et al. 1993b). Each MBP/G fusion protein was loca-
lized in the periplasm as determined by SDS-PAGE and western
5   blotting with anti-MBP antisera (data not shown). Hemaggluti-
nation (HA) assays were performed on the cells co-expressing
the pap operon with either MBP (pMAL-p4) or with each of the
MBP/G fusions. Co-expression of the MBP/G1'-140' fusions with
the pap operon decreased the HA titer 30 fold compared to the
10 MBP control (see table 3).

TABLE 3: MBP/G fusions inhibit P pilus formation

MBP/ proteins[a]	MBP	MBP/G1'-19'	MBP/G1'-81'	MBP/G1'-140'
HA titer[b]	128	64	82	4
Piliation percentage[c]	100%	50%	40%	10%

15 [a] Plasmids pMAL-p4, pMAL/G1'-19', pMAL/G1'-81' and pMAL/G1'-140' encoding
MBP, MBP-G1'-19', MBP-G1'-81' and MBP-G1'-140', respectively, were
transformed into the strain HB101/pFJ3 and the expression of pap operon
and each of the MBP/G fusions was induced in each strain.
[b] Highest dilution of cell suspension yielding detectable HA
20 [c] The pili preparations were analyzed on Coomassie blue stained gels and
the PapA bands were scanned by a densitometer. The relative piliation was
determined by equating the densitometric value of PapA to 100% of the
control. (HB101/pFJ3/pMAL-p4).

Co-expression of the MBP/G1'-81' and MBP/G1'-19' fusions with
25 the pap operon had only weak effects on the HA titer (Table
3). Co-expression of MBP/G1'-140' with the pap operon reduced
the amount of pili that could be purified from the cells by
90% whereas MBP/G1'-19' and MBP/G1'-81' reduced the amount of
pili formed by about 50% compared to MBP alone (Table 3).
30 Electron microscopy confirmed that cells expressing the
MBP/G1'-140' fusion had little or no pili compared to the
fully piliated cells co-expressing MBP (data not shown).

We hypothesized that the co-expression of the MBP/G1'-140'
fusion with the pap operon inhibited pilus formation by

titrating *PapD* away from the subunits thus driving subunits
down dead end pathways of aggregation and proteolytic degra-
dation (Hultgren et al., 1989; Holmgren et al., 1992). This
hypothesis was tested as follows. pMAL-p4, pMAL/G1'-19',
pMAL/G1'-81' or pMAL/G1'-140' were transformed into BH101
carrying pFJ22 (Jacob-Dubuisson et al., 1994). Plasmid pFJ22
encodes *papDJKEFGA* under the control of the P_{tac} promoter.
In HB101/pFJ22, pilus subunits accumulate in the periplasmic
space due to the absence of the usher, PapC. The effect of
expressing each fusion on the fate of each pilus subunit type
was determined by western blotting (Fig. 22). A significant
decrease in the amount of *PapG*, PapA and PapF in cells co-
expressing the MBP/G1'-140' fusion was detected arguing that
the MBP/G1'-140' fusion blocked chaperone-subunit complex
formation by interacting with *PapD* and trapping it away from
pilus subunits. There was no effect on PapK and PapE stabi-
lity for reasons that are not understood. Nevertheless, the
fusion was capable of blocking pilus formation by interfering
with the formation of critical chaperone-subunit complexes.

In vitro chaperone binding assays

PapD binding to the MBP/G fusion proteins was further inves-
tigated using two different *in vitro* assays. In the first
assay, purified MBP/G fusion proteins or MBP, were incubated
with *PapD* and complex formation was analyzed on silver
stained IEF gels. *PapD* bound to the MBP/G1'-140' fusion
protein and formed a stable complex that migrated to an
identical pI (5.2) as that seen for the complex formed *in
vivo* (Fig. 23A, lane 4). In contrast, no complexes between
PapD and MBP/G1'-81', MBP/G1'-19' or the MBP control were
detected (Fig. 23A, lane 1, 2 and 3). In the second assay, we
investigated the ability of *PapD* to bind to the three differ-
ent MBP/G fusion proteins immobilized on microtiter wells and
quantitated the interactions in an enzyme-linked immunosor-
bent assay (ELISA) using anti-*PapD* antiserum (Fig. 23B). *PapD*
bound weakly to MBP/G1'-19', slightly better to MBP/G1'-81'
but very strongly to the MBP/G1'-140'. *PapD* did not bind the

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MBP control. The dramatic increase in the affinity of PapD
for the MBP/G1'-140' fusion as compared to the MBP/G1'-81'
and MBP/G1'-19' fusions, suggested that residues 81' to 140'
in PapG were critical for strong PapD binding.

5   The invariant cleft residues Arg-8 and Lys-112 in PapD have
been shown to form a molecular anchor in the chaperone cleft
necessary for binding pilus subunits (Slonim et al., 1992;
Kuehn et al., 1993). Mutations in these residues abolished or
greatly reduced the ability of PapD to bind subunits and
10   mediate pilus assembly (Kuehn et al., 1993). Arg-8A and Lys-
112A mutant PapD proteins demonstrated a greatly reduced
ability to bind to the MBP/G1'-140' fusion protein both in
vivo and in vitro (data not shown). These results argue that
the interactions between PapD and the MBP/G1'-140' fusion are
15   biologically relevant.

The upstream site is an independent binding determinant

We tested the ability of PapD to bind three PapG COOH-ter-
minal truncate proteins (shown in Fig. 20) in order to deli-
neate the limits of the upstream PapD binding site and to
20   test whether it was capable of functioning as an independent
site. The truncate proteins were co-expressed with PapD in
strain KS474 (Strauch et al., 1989) which carries a kanamycin
cassette in the degP locus (degP41 mutant). Both the PapG2
and PapG3 truncates were expressed and stable in KS474,
25   however the PapG1 truncate (removing the last 14 residues)
underwent limited degradation (data not shown). Complex
formation was assayed by analyzing periplasmic extracts on
acid native polyacrylamide gels (Striker et al., 1994). Anti-
PapD and anti-PapG antisera was used to detect the PapD-PapG
30   complexes after western blotting (Fig. 24). PapD-PapG com-
plexes are resolved from PapD alone on these gels due to
their differences in size, shape and charge (Striker et al.,
1994) PapD formed a complex with full-length PapG as well as
with the PapG2 truncate (Fig. 24A, lanes 2 and 3). The com-
35   plex band was also recognized by anti PapG antiserum (Fig.

24B, lanes 2 and 3). PapD did not form a complex with the
PapG3 truncate (Fig. 24A & B, lane 4) which terminates at
amino acid 145. This information delineates an endpoint for
the second PapD binding site (as indicated in Fig. 20).

5   Combining the PapG truncate and the MBP/G fusion data (shown
in Fig. 20) suggested that the second site on PapG recognized
by PapD resided in a region between residues 117' to 141' of
PapG. Four overlapping peptides were synthesized correspon-
ding to the region between residues 120' to 156' and tested
10   for their ability to bind PapD in an ELISA assay. Such a
strategy proved successful in studying the COOH-terminal PapD
binding site (Kuehn et al., 1993). The peptide corresponding
to residues 125' to 140', but not the other three peptides,
bound to PapD in the ELISA (Fig. 25). These data argue that
15   PapD recognizes two surfaces on PapG. PapD forms a beta
strand zippering interaction with the COOH-terminus but also
recognizes a region containing residues 125'-140'.

Discussion and conclusions

In conclusion, a chaperone binding assay was developed using
20   fusions of the carboxyl terminus of PapG to Maltose Binding
Protein (MBP/G fusions) to investigate whether chaperone-
subunit complex formation requires additional interactions.
PapD bound strongly to an MBP/G fusion containing the C-ter-
minal 140 amino acids of PapG (MBP-G1'-140') but only weakly
25   to the MBP-G1'-81', arguing that the region between the C-
terminal residues 81' and 140' contains additional infor-
mation that is required for strong PapD-PapG interactions.
PapD was further shown to interact with a PapG C-terminal
truncate containing residues 117'-314' (corresponding to a
30   truncate consisting of the first 198 N-terminal amino acid
residues of PapG), but not with a truncate containing resi-
dues 170'-314' (corresponding to a truncate consisting of the
first 145 N-terminal amino acid residues of PapG).

These results together suggest that a second, independent
PapD interactive site exists which does not interact with the
C-terminus of the pilus subunit. This assumption is further
confirmed by the fact that one of four peptides overlapping
5   the second site region of PapG was recognized by PapD. The
last result also rules out that the function of the upstream
site was to exert secondary effects on the COOH terminal
chaperone binding site since this region was independently
capable of facilitating PapD binding.

10   Unlike what is true for the native subunits it was demon-
strated that MBP-G1'-140' is capable of folding even in the
absence of PapD and remain soluble in the periplasmic space;
this folding involves the formation of an intramolecular
disulphide bond. This is consistent with earlier data showing
15   that the subunits themselves are highly folded when bound to
PapD (Kuehn et al., 1991; Striker et al., 1994). However, it
is not yet known whether PapD binds to subunits in vivo after
they fold or whether folding occurs in the context of an
interaction with PapD.

20   Finally, the expression of the MBP/G fusions in cells produ-
cing P pili inhibited pilus assembly to varying degrees by
binding to PapD and preventing chaperone-subunit complex
formation. The ability of the fusions to block pilus assembly
increased as the length of the fusion protein increased.
25   MBP-G1'-140' was a strong inhibitor of pilus assembly while
MBP-G1'-81' and MBP-G1'-19' were weak inhibitors. This is
consistent with the finding that residues 140'-81' contain a
region necessary for strong interactions between PapD and
PapG.

30   Recent research by the inventors has further revealed that
the simultaneous expression in vivo of PapG and a modified
PapD polypeptide free of domain 1 in a degP41 strain (KS474)
expressing this truncated PapD results in 1) suppression of
PapG toxicity in this cell line and 2) efficient partitioning

into the periplasmic space of PapG. However, the pilus sub-
unit is not correctly folded after release from PapD.

It is thus suggested by the inventors, that the binding
between pilus subunits and PapD takes place by the pilus
5   subunit binding to domain 1 and 2 of PapD. The binding
between the subunit and domain 1 is important for the correct
folding of the subunit, and the binding between the subunit
and domain 2 is important for the transport of the subunit
out into the periplasm. Whether domain 2 of PapD also par-
10 ticipates in the folding of subunits is not known.

Since the above results are strong indicators of the exis-
tence of at least one binding site apart from the one invol-
ving Arg-8 and Lys-112 of PapD, and since it seems that this
novel binding site is also very important in the net inter-
15 action between PapD and the pilus subunits, it is contem-
plated that also effecting this binding site will have the
antibacterial effects described herein.

It is thus the plan to elucidate the motif of binding between
this second binding site of PapD in manners similar to those
20 described herein, and it is further the plan to
design/identify compounds capable of interacting with this
second binding site in order to ultimately synthesize com-
pounds capable of interacting with this site in such a manner
that assembly of intact pili is prevented, inhibited or
25 enhanced.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: Washington University
        (B) STREET: 724 South Euclide Avenue
        (C) CITY: St. Louis
        (D) STATE: Missouri
        (E) COUNTRY: USA
        (F) POSTAL CODE: MO 63110

    (i) APPLICANT:
        (A) NAME: Symbicom Aktiebolag
        (B) STREET: Tvistevaegen 48
        (C) CITY: Umea
        (E) COUNTRY: Sweden
        (F) POSTAL CODE: S-907 36 Umea

    (ii) TITLE OF INVENTION: TREATMENT OR PROPHYLAXIS OF DISEASES CAUSED
        BY PILUS-FORMING BACTERIA

    (iii) NUMBER OF SEQUENCES: 27

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

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        (A) LENGTH: 874 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (iii) HYPOTHETICAL: NO

    (iii) ANTI-SENSE: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Escherichia coli

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        (A) NAME/KEY: CDS
        (B) LOCATION: 1..720

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        (A) NAME/KEY: sig_peptide
        (B) LOCATION: 1..63

    (ix) FEATURE:
        (A) NAME/KEY: mat_peptide

169

(B) LOCATION: 64..717

(x) PUBLICATION INFORMATION:
    (A) AUTHORS: Norgren, M.
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                    Normark, S.
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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GAC GGG AGT GAG AAG TCA ATG ACG CTT GAT ATC TCC AAT GAT AAC AAA       144
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CAA CTG CCC TAT CTT GCT CAG GCA TGG ATA GAA AAT GAA AAT CAG GAA       192
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AAA ATT ATT ACA GGG CCG GTT ATT GCC ACC CCT CCG GTT CAG CGC CTT       240
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  Thr Pro Tyr Leu Ser Tyr Ile Asn Asp Tyr Gly Gly Arg Pro Val Leu
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(2) INFORMATION FOR SEQ ID NO: 2:

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         (B) TYPE: amino acid
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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            15              20              25

Gln Leu Pro Tyr Leu Ala Gln Ala Trp Ile Glu Asn Glu Asn Gln Glu
         30              35              40

Lys Ile Ile Thr Gly Pro Val Ile Ala Thr Pro Pro Val Gln Arg Leu
    45              50              55

Glu Pro Gly Ala Lys Ser Met Val Arg Leu Ser Thr Thr Pro Asp Ile
 60              65              70                      75

Ser Lys Leu Pro Gln Asp Arg Glu Ser Leu Phe Tyr Phe Asn Leu Arg
                80              85              90

Glu Ile Pro Pro Arg Ser Glu Lys Ala Asn Val Leu Gln Ile Ala Leu
             95              100             105
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Gln Thr Lys Ile Lys Leu Phe Tyr Arg Pro Ala Ala Ile Lys Thr Arg
　　　110　　　　　　　　　　115　　　　　　　　　120

Pro Asn Glu Val Trp Gln Asp Gln Leu Ile Leu Asn Lys Val Ser Gly
　　125　　　　　　　　130　　　　　　　　135

Gly Tyr Arg Ile Glu Asn Pro Thr Pro Tyr Tyr Val Thr Val Ile Gly
140　　　　　　　　145　　　　　　　　150　　　　　　　　155

Leu Gly Gly Ser Glu Lys Gln Ala Glu Glu Gly Glu Phe Glu Thr Val
　　　　　　160　　　　　　　　165　　　　　　　170

Met Leu Ser Pro Arg Ser Glu Gln Thr Val Lys Ser Ala Asn Tyr Asn
　　　　175　　　　　　　　180　　　　　　　　185

Thr Pro Tyr Leu Ser Tyr Ile Asn Asp Tyr Gly Gly Arg Pro Val Leu
　　　　190　　　　　　　　195　　　　　　　200

Ser Phe Ile Cys Asn Gly Ser Arg Cys Ser Val Lys Lys Glu Lys
　　205　　　　　　　　210　　　　　　　　215

(2) INFORMATION FOR SEQ ID NO: 3:

　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　(A) LENGTH: 30 base pairs
　　　　　(B) TYPE: nucleic acid
　　　　　(C) STRANDEDNESS: single
　　　　　(D) TOPOLOGY: linear

　　　(ii) MOLECULE TYPE: DNA (Synthetic)

　　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GTCAAACACC GCCGGAACTC GTCCAGGCGA　　　　　　　　　　　　　　　　　　30

(2) INFORMATION FOR SEQ ID NO: 4:

　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　(A) LENGTH: 30 base pairs
　　　　　(B) TYPE: nucleic acid
　　　　　(C) STRANDEDNESS: single
　　　　　(D) TOPOLOGY: linear

　　　(ii) MOLECULE TYPE: DNA (Synthetic)

　　　(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CGGGCGATAA AAAAGAGCTA TTTTGGTCTG　　　　　　　　　　　　　　　　　　30

(2) INFORMATION FOR SEQ ID NO: 5:

　　　(i) SEQUENCE CHARACTERISTICS:
　　　　　(A) LENGTH: 27 base pairs
　　　　　(B) TYPE: nucleic acid
　　　　　(C) STRANDEDNESS: single

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        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (Synthetic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCGATAAAAA AGCATTATTT TCCTCTG                                             27


(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Gly Lys Arg Lys Pro Gly Glu Leu Ser Gly Ser Met Thr Met Val Leu
1               5                   10                  15

Ser Phe Pro


(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 16 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Lys Pro Gly Glu Leu Ser Gly Ser Met Thr Met Val Leu Ser Phe Pro
 1               5                   10                  15


(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 11 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide


    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ser Gly Ser Met Thr Met Val Leu Ser Phe Pro
1               5                   10
```

(2) INFORMATION FOR SEQ ID NO: 9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 7 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

    Thr Met Val Leu Ser Phe Pro
    1   5

(2) INFORMATION FOR SEQ ID NO: 10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

    Gly Lys Arg Lys Pro Val Glu Leu Ser Gly Ser Met Thr Met Val Leu
    1   5   10   15

    Ser Ser Pro

(2) INFORMATION FOR SEQ ID NO: 11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 19 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

    Gly Lys Arg Lys Pro Gly Glu Leu Ser Gly Ser Met Thr Met Val Leu
    1   5   10   15

    Ser Phe Pro

(2) INFORMATION FOR SEQ ID NO: 12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 amino acids
        (B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Glu Glu Gly Lys Arg Lys Pro Gly Glu Leu Ser Gly Ser Met Thr Met
1   5   10   15

Val Leu Ser Phe
        20

(2) INFORMATION FOR SEQ ID NO: 13:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 19 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Gln Asn Leu Ile Ala Gly Pro Phe Ser Ala Thr Ala Thr Leu Val Ala
1   5   10   15

Ser Tyr Ser

(2) INFORMATION FOR SEQ ID NO: 14:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 19 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Lys Lys Leu Glu Ala Gly Asn Tyr Phe Ala Val Leu Gly Phe Arg Val
1   5   10   15

Asp Tyr Glu

(2) INFORMATION FOR SEQ ID NO: 15:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 19 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Lys Ser Val Val Pro Gly Asp Tyr Glu Ala Thr Ala Thr Phe Glu Leu
1   5   10   15

Thr Tyr Arg

(2) INFORMATION FOR SEQ ID NO: 16:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 19 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Gly Ile Leu Asn Gly Gly Asp Phe Gln Thr Thr Ala Ser Met Ala Met
1   5   10   15

Ile Tyr Asn

(2) INFORMATION FOR SEQ ID NO: 17:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 16 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Tyr Ala Leu Ala Pro Asn Ala Val Ile Pro Thr Ser Leu Ala Leu Leu
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 18:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 19 amino acids
            (B) TYPE: amino acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Lys Ser Val Val Pro Gly Asp Tyr Glu Ala Thr Ala Thr Phe Glu Leu
1   5   10   15

Thr Tyr Arg

(2) INFORMATION FOR SEQ ID NO: 19:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 16 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Leu Pro Ala Thr Asn Thr Leu Met Leu Ser Phe Asp Asn Val Gly Gly
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 20:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 16 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Asp Gln Ile Lys Gln Leu Pro Ala Thr Asn Thr Leu Met Leu Ser Phe
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 21:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 16 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Tyr Lys Met Pro Tyr Asp Gln Ile Lys Gln Leu Pro Ala Thr Asn Thr
1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 15 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

    Gln His His Tyr Tyr Asp Leu Trp Gln Asp His Tyr Lys Met Pro Tyr
    1   5   10   15

(2) INFORMATION FOR SEQ ID NO: 23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 33 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (synthetic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

CCCCCCTGCA GATCAGATTA AGCAGCTACC TGC   33

(2) INFORMATION FOR SEQ ID NO: 24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

CCCCTGCAGT AAAAATATCT CTGCTCAGAA ATAC   34

(2) INFORMATION FOR SEQ ID NO: 25:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 28 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (synthetic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CCATCGATGA ACAGCCAGTC AGATAATC   28

(2) INFORMATION FOR SEQ ID NO: 26:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 61 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (synthetic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GGAAAGAGAA AACCCGGGGA GCTATCTGGT TCTATGACTA TGGTTCTGAG TTTCCCCTCA   60

T   61

(2) INFORMATION FOR SEQ ID NO: 27:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 67 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (synthetic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

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CGATCAGGGG AAACTCAGAA CCATAGTCAT AGAACCAGAT AGCTCCCGGG GTTTTCTCTT   60

TCCTGCA   67

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CLAIMS

1. A method for the treatment and/or prophylaxis of diseases
caused by tissue-adhering pilus-forming bacteria, comprising
preventing, inhibiting or enhancing binding between at least
5   one type of pilus subunit and at least one type of periplas-
mic molecular chaperone in the pilus-forming bacteria, the
periplasmic molecular chaperone being one that binds pilus
subunits during transport of these pilus subunits through the
periplasmic space and/or during the process of assembly of
10   the intact pilus.

2. A method according to claim 1, comprising administering,
to a subject in need thereof, an effective amount of a sub-
stance capable of interacting with at least one type of
periplasmic molecular chaperone which binds pilus subunits
15   during transport of these pilus subunits through the peri-
plasmic space and/or during the process of assembly of the
intact pilus, in such a manner that binding of pilus subunits
to the periplasmic molecular chaperone is prevented,
inhibited or enhanced.

20   3. A method according to claim 1 or 2, wherein the bacteria
are selected from the group consisting of *Haemophilus* spp,
Helicobacter spp, *Pseudomonas aeruginosa*, *Mycoplasma* spp, and
all members of the *Enterobacteriacieae* family, including
Escherichia spp, *Salmonella* spp, *Bordetella* spp, *Yersinia*
25   spp, *Klebsiella* spp., and *Proteus* spp.

4. A method according to any of claims 1-3, wherein the
periplasmic molecular chaperone is a periplasmic protein
selected from the group consisting of *PapD*, *FimC*, *SfaE*, *FaeE*,
FanE, *Cs3-1*, *F17D*, *ClpE*, *EcpD*, *Mrkb*, *FimB*, *SefB*, *HifB*, *MyfB*,
30   *PsaB*, *PefD*, *YehC*, *MrpD*, *CssC*, *NfaE*, *AggD*, and *Caf1M*.

5. A method according to any of claims 1-4, wherein the
prevention, inhibition or enhancement of the binding is
accomplished by interaction with, in the periplasmic molecu-

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lar chaperone, a binding site which is normally involved in
binding to pilus subunits during transport of these pilus
subunits through the periplasmic space and/or during the
process of pilus assembly.

5   6. A method according to claim 5, wherein the binding site is
     a binding site to which the carboxyl terminal part of a pilus
     subunit is capable of binding, and which comprises site
     points substantially identical to the invariant residues
     Arg-8 and Lys-112 in PapD, and a polypeptide fragment which
10   is capable of interacting with a β-strand of the carboxyl
     terminal part of the pilus subunit, thereby stabilizing the
     binding of said subunit at the Arg-8 and Lys-112 site points
     of the binding site.

     7. A method according to claim 5, wherein the binding site is
15   one to which the peptide G125'-140' and/or the fusion peptide
     MBP-G1'-140' and/or the peptide G1'-19'is capable of binding.

     8. A method for identifying a potentially therapeutically
     useful substance capable of interacting with a periplasmic
     molecular chaperone, thereby preventing, inhibiting or enhan-
20   cing the interaction between a periplasmic molecular chape-
     rone and a pilus subunit, the method comprising at least one
     of the following steps:

     1) testing a candidate substance in an assay in which the
     possible prevention, inhibition or enhancement by the sub-
25   stance of the interaction between the periplasmic molecular
     chaperone and the pilus subunit is determined by

         adding the substance to a system comprising the periplasmic
         molecular chaperone or an analogue thereof in an immobi-
         lized form and the pilus subunit or an equivalent thereof
30   in a solubilized form and determining the change in binding
         between the pilus subunit or equivalent thereof and the
         periplasmic molecular chaperone or analogue thereof caused
         by the addition of the substance, or

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adding the substance to a system comprising the pilus subunit or an equivalent thereof in an immobilized form and the periplasmic molecular chaperone or an analogue thereof in a solubilized form and determining the change in binding
5   between the pilus subunit or equivalent thereof and the periplasmic molecular chaperone or analogue thereof caused by the addition of the substance, or

adding the substance to a system comprising the pilus subunit or an equivalent thereof as well as the periplasmic
10   molecular chaperone or an analogue thereof in solubilized form and determining the change in binding between the pilus subunit or equivalent thereof and the periplasmic molecular chaperone or analogue thereof caused by the addition of the substance, or

15   adding the substance to a system comprising the pilus subunit or an equivalent thereof as well as the periplasmic molecular chaperone or an analogue thereof in solubilized form and measuring the change in binding energy caused by the addition of the substance, and identifying the sub-
20   stance as potentially therapeutically useful if a signifi- cant change in the binding energy between the pilus subunit or equivalent thereof and the periplasmic molecular chape- rone or analogue thereof is observed,

and identifying the substance as potentially therapeutically
25   useful if a significant change in the binding or binding energy between the pilus subunit or equivalent thereof and the periplasmic molecular chaperone or analogue thereof is observed;

2)   testing a candidate substance in an assay in which the
30   possible prevention, inhibition or enhancement of the inter- action between the periplasmic molecular chaperone and the pilus subunit is determined by

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adding the substance to a system comprising living tissue-
adhering pilus-forming bacteria followed by determination
of the growth rate of the bacteria, a reduction in growth
rate compared to a corresponding system wherein the sub-
stance has not been added being indicative of prevention,
inhibition or enhancement of the binding between the peri-
plasmic molecular chaperone and the pilus subunit, or

adding the substance to a system comprising living tissue-
adhering pilus-forming bacteria followed by a determination
of the tissue adhesion of the bacteria, a reduction in
tissue adhesion compared to a corresponding system wherein
the substance has not been added being indicative of pre-
vention, inhibition or enhancement of the binding between
the periplasmic molecular chaperone and the pilus subunit,

and identifying the substance as potentially therapeutically
useful if a reduction in growth rate or tissue adhesion is
observed after the addition of the substance; and

3)   administering, to an experimental animal, a substance
        which has been established *in vitro* to prevent, inhibit
        or enhance the interaction between a periplasmic mole-
        cular chaperone and a pilus subunit, the experimental
        animal being inoculated with tissue-adhering pilus-
        forming bacteria before, simultaneously with or after
        the administration of the substance, and electing as a
        substance suitably capable of interacting with a peri-
        plasmic molecular chaperone, a substance preventing
        and/or curing and/or alleviate disease caused by the
        bacteria.

9. A method according to claim 8, wherein the assay in step 1
comprises

- adding the substance to a first system comprising the
periplasmic molecular chaperone or an analogue thereof,

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- subsequently adding a pilus subunit or an equivalent there-
of which has been labelled with an environmentally sensitive
fluorescent probe,

- determining the fluorescent emission at a particular wave-
5   length which is indicative of the amount of binding between
the periplasmic molecular chaperone or the analogue thereof
and the pilus subunit or the equivalent thereof, and

- comparing the determined fluorescent emission to fluores-
cent emission determined in a corresponding second system
10   containing substantially the same concentrations of the
molecular chaperone or the analogue thereof and the pilus
subunit or the equivalent thereof but substantially no sub-
stance,

a significant difference in fluorescent emission between the
15   first and second system being indicative of interaction
between the periplasmic molecular chaperone or the analogue
thereof and the substance.

10. A method according to claim 9, wherein the determination
of fluorescent emission in the second system is performed a
20   plurality of times at varying molar ratios between the pilus
subunit or the equivalent thereof and the periplasmic chape-
rone and the equivalent thereof, whereupon the constant of
binding between the pilus subunit or equivalent thereof and
the periplasmic molecular chaperone or analogue thereof is
25   assessed from the determined fluorescent emission data.

11. A method according to claim 9 or 10, wherein the determi-
nation of fluorescent emission in the first system is per-
formed a plurality of times at varying molar ratios between
the substance and the periplasmic chaperone and the equiva-
30   lent thereof, whereafter the constant of binding between the
substance and the periplasmic molecular chaperone or analogue
thereof is assessed from the determined fluorescent emission
data.

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12. A method according to any of claims 8-11, wherein the equivalent of the pilus subunit is G1'-19'WT, MBP-G1'-140', or G125'-140' and the periplasmic molecular chaperone is PapD.

5   13. A method according to any of claims 8-12, wherein the living, tissue adhering pilus-forming bacteria are of a protease deficient strain, the protease being one which is at least partially responsible for the degradation of pilus subunits

10 14. A method according to claim 13, wherein the protease deficient strain is a degP41 strain.

15. A method for identifying and/or designing a substance, X, capable of interacting with a periplasmic molecular chapero-ne, e.g. binding to the periplasmic molecular chaperone, with
15 a predicted binding free energy equal to or better than a predetermined threshold value, the method comprising

1)   selecting a substance, A, which could potentially inter-
     act with a site in the periplasmic molecular chaperone,
     and providing a 3-dimensional structural representation
20   thereof,

2)   predicting the binding free energy between the substance
     A and the site in the periplasmic molecular chaperone or
     a site analogous to such a site,

25 3) if the predicted binding free energy between the sub-
        stance A and the site in the periplasmic molecular chape-
        rone or the analogous site is equal to or better than the
        predetermined threshold value, then identifying the
        substance A as the substance X,

30 4) if the predicted binding free energy between the sub-
        stance A and the site in the periplasmic molecular chape-
        rone or the analogous site is not equal to or better than

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a predetermined threshold value, then modifying the 3-dimensional structural representation and predicting the binding free energy between the thus modified substance, B, and the site in the periplasmic molecular chaperone or
5   the analogous site, and

5)   repeating step 4 until the predicted binding free energy
     determined between the resulting substance, X, and the
     site in the periplasmic molecular chaperone or the ana-
     logous site is equal to or better than the predetermined
10   threshold value.

16. A method for identifying and/or designing a substance, Y,
capable of interacting with a periplasmic molecular chapero-
ne, e.g. binding to the periplasmic molecular chaperone, with
a binding free energy equal to or better than a predetermined
15   threshold value, the method comprising

1)   Identifying a substance, X, according to the method of
     claim 15, and

2)   providing a sample of the chemical substance X and a
     sample of the periplasmic molecular chaperone or an
20   analogue thereof and measuring the binding free energy
     between the chemical substance X and the periplasmic
     molecular chaperone or analogue thereof, and establishing
     that the measured binding free energy between the chemi-
     cal substance X and the periplasmic molecular chaperone
25   or analogue thereof is equal to or better than the prede-
     termined threshold value, and then identifying the sub-
     stance X as the substance Y, and optionally

3)   subjecting the substance Y to the method according to any
     of claims 8-14 to verify that the substance Y is a poten-
30   tially therapeutically useful substance capable of inter-
     acting with a periplasmic molecular chaperone.

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17. A method according to claim 15 or 16, wherein the binding free energy is predicted by

assessing the average energy difference, $<\Delta V_{X\text{-}s}^{el}>$, defined as $<V_{X\text{-}s}^{el}>_B - <V_{X\text{-}s}^{el}>_A$, between the contribution from polar inter-
5 actions to the potential energy between the chemical substance X and its surroundings (denoted s) in two states, one state (A) being where the chemical substance is surrounded by solvent, the other state (B) being where the chemical substance, bound to the site in the periplasmic molecular chape-
10 rone or bound to a site analogous to such a site, is surrounded by solvent,

assessing the average energy difference, $<\Delta V_{X\text{-}s}^{vdw}>$, defined as $<V_{X\text{-}s}^{vdw}>_B - <V_{X\text{-}s}^{vdw}>_A$, between the contribution from non-polar interactions to the potential energy between the chemical
15 substance X and its surroundings (denoted s) in two states, one state (A) being where the chemical substance is surrounded by solvent, the other state (B) being where the chemical substance, bound to the site in the periplasmic molecular chaperone or bound to a site analogous to such a
20 site, is surrounded by solvent, and

calculating the absolute binding free energy as an adjusted combination of the two above-mentioned average energy differences.

18. The method according to claim 17, wherein the site in the
25 periplasmic molecular chaperone is the pilus subunit binding part of a periplasmic molecular chaperone, such as the pilus subunit binding site of a periplasmic molecular chaperone selected from the group consisting of *PapD*, *FimC*, *SfaE*, *FaeE*, *FanE*, *Cs3-1*, *F17D*, *ClpE*, *EcpD*, *Mrkb*, *FimB*, *SefB*, *HifB*, *MyfB*,
30 *PsaB*, *PefD*, *YehC*, *MrpD*, *CssC*, *NfaE*, *AggD* and *CaflM*, or an analogue of such a pilus subunit binding site.

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19. The method according to claim 18, wherein the site in the periplasmic molecular chaperone is a pilus subunit binding site of PapD or an analogue thereof.

20. The method according to any of claims 15-19, wherein the
5   substance A is chosen by performing the following steps:

-   co-crystallizing the periplasmic molecular chaperone or
    the analogue thereof with a ligand capable of interacting
    with a site in the periplasmic molecular chaperone or the
    analogue thereof and establishing the three-dimensional
10 conformation of the periplasmic molecular chaperone or
    the analogue thereof and the ligand when interacting by
    means of X-ray crystallography,

-   using the above-established conformation of the periplas-
    mic molecular chaperone or the analogue thereof to estab-
15 lish a 3-dimensional representation of the site in the
    periplasmic molecular chaperone or the analogue thereof
    interacting with the ligand during binding,

-   selecting a number of distinct chemical groups, X1, and
    determining the possible spatial distributions of the X1
20 chemical groups which maximizes the binding free energy
    between the chemical groups and the site in the chaperone
    or the analogue interacting with the ligand,

-   extracting, from a database comprising three-dimensional
    representations of molecules, a molecule which has the X1
25 chemical groups in the possible spatial distributions
    determined above,

-   optionally modifying the 3-dimensional representation of
    the molecule extracted from the database, and

    identifying the optionally modified molecule as the substance
30 A.

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21. A method according to claim 20, wherein the ligand is a pilus subunit or a part thereof with which the periplasmic molecular chaperone normally interacts during transport of the pilus subunit through the periplasmic space and/or during
5 pilus assembly.

22. A novel compound of the general formula:

I
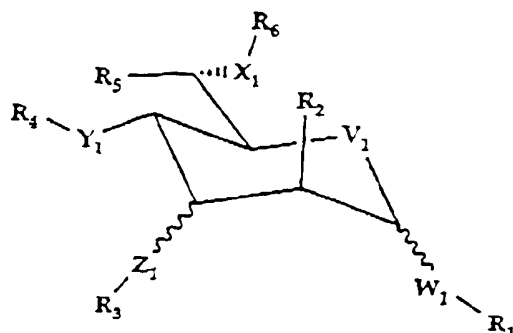
wherein

V_1 is O, S, SO, SO_2, CH_2, C(OH)H, CO or CS;

10 W_1 is O, S, SO_2, SO_3, CH_2 or NH;

R_1 is H; C_{1-24} alkyl, C_{1-24} alkenyl or C_{1-24} alkynyl, which alkyl, alkenyl and alkynyl may be substituted with one or more substituents independently selected from OH, $-CONH_2$, $-CSNH_2$, $-CONHOH$, $-CSNHOH$, $-NHCHO$, $-NHCONH_2$, $-NHCSNH_2$,
15 $-NHSO_2NH_2$ and $-SO_2NH_2$; acyl; or $-(CH_2CH_2O)_s-H$, wherein s = 1,2,3;

R_2 is a group of the formula

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wherein

   A is $-CH-(CH_2)_n-$, or $-CH=CH-(CH_2)_{n-1}-$ (n>0) or
     $-CH-(CH_2)_n-$;
        ‖
       Y_2

   B is $-(CH_2)_m-$ or $=CH-(CH_2)_{m-1}-$ (m>0);

   X_2 is N, CH or C (when B is $=CH-(CH_2)_{m-1}-$; and

   Y_2 is O, S, NH, H_2 or H (n=1); and

   4>m+n>0, n<3, and m<3;

or R_2 is a group of the formula

wherein

   A is $-CH-(CH_2)_n-$, or $-CH=CH-(CH_2)_{n-1}-$ (n>0) or
   $-CH-(CH_2)_n-$;
      ‖
     Y_2

   B is $-(CH_2)_m-$ or $=CH-(CH_2)_{m-1}-$ (m>0); and

   X'_2 is O, NH, CH_2 or S (when p=0); N or CH (p=1); or C
   (when p=1 and B is $=CH-(CH_2)_{m-1}-$);

   V_2, Z_2 and W_2 are independently H, OH, $-CONH_2$, $-CSNH_2$,
   $-CONHOH$, $-CSNHOH$, $-NHCHO$, $-NHCONH_2$, $-NHCSNH_2$,
   $-NHSO_2NH_2$, $-SO_2NH_2$, or V_2 and Z_2, or Z_2 and W_2 together
   form $-NHC(O)NH-$, $-C(O)NHC(O)-$, $-NHS(O_2)NH-$, $-C(O)NHO-$,
   $-C(S)NHO-$, $-S(O_2)NHO-$, or $-S(O_2)NHC(O)-$;
   4>m+n>0, n<3, and m<3;

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or R_2 is a group $-W_5-(C_{1-5}$ alkyl or C_{2-5} alkenyl or C_{2-5} alkynyl) wherein W_5 is a bond or is selected from $-O-$, $-S-$, $-SO_2-$, and $-NHC(O)-$, and the C_{1-5} alkyl, C_{2-5} alkenyl or C_{2-5} alkynyl moiety may be substituted with up to three groups
5   selected independently from OH, $-CONH_2$, $-CSNH_2$, $-CONHOH$, $-CSNHOH$, $-NHCHO$, $-NHCONH_2$, $-NHCSNH_2$, $-NHSO_2NH_2$ and $-SO_2NH_2$;

$-Z_1-R_3$ is $-SO_2(OH)$, $-PO(OH)_2$, $-OSO_2(OH)$, $-NHSO_2(OH)$, $-SPO(OH)_2$, $-CH_2COOH$, tetrazol-5-yl or tetrazol-5-ylmethyl, or salts thereof;

10   or Z_1 is $-O-$, $-S-$, $-NH-$, or $-CH_2-$, and R_3 is a group of the formula:
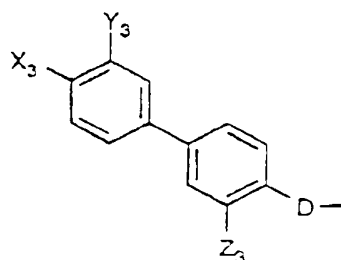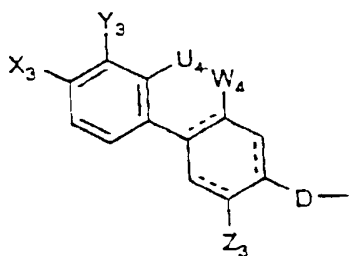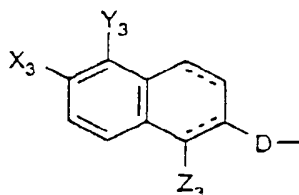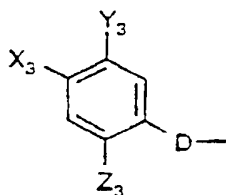
D is $-CH_2-$, $-CO-$, $-SO_2-$, $-NH-SO_2-$, $-NH-CO-$, $-O-PO(OH)-$ or a salt thereof;

Z_3 is H, OH, $-CONH_2$, $-CSNH_2$, $-CONHOH$, $-CSNHOH$, $-NHCHO$,
15    $-NHCONH_2$, $-NHCSNH_2$, $-NHSO_2NH_2$, $-SO_2NH_2$, $-SO_2(OH)$, $-PO(OH)_2$, $-OSO_2(OH)$, $-NHSO_2(OH)$, $-COOH$, tetrazolyl-5-yl or tetrazolyl-5-ylmethyl or a salt thereof,

with the proviso that when D is -CH$_2$-, -CO-, -SO$_2$-, -NHSO$_2$-
or -NHCO-, then Z$_3$ is -SO$_2$(OH), -PO(OH)$_2$, -OSO$_2$(OH),
-NHSO$_2$(OH), -COOH, tetrazolyl-5-yl or tetrazolyl-5-ylmethyl
or a salt thereof;

5   X$_3$ and Y$_3$ independently are H, NO$_2$, SO$_2$NH$_2$, CONH$_2$, CF$_3$ or F;
and

U$_4$-W$_4$ is -CHCH-, -CH$_2$CH$_2$-, -C(OH)CH-, -CHC(OH)-,
-CH(OH)CH$_2$-, -CH$_2$CH(OH)-, -CH(OH)CH(OH)-, -C(O)NH-,
-NHC(O)-;

10   Y$_1$ is -O- or -S-;

R$_4$ is H or, when Y$_1$ is S, S(CH$_2$)$_q$N(R$_9$)$_3$$^+$ and q is an integer
2-4, where R$_9$ is H or CH$_3$;

R$_5$ is H; C$_{1-6}$ alkyl, C$_{2-6}$ alkenyl or C$_{2-6}$ alkynyl, and the C$_{1-6}$
alkyl, C$_{2-6}$ alkenyl or C$_{2-6}$ alkynyl moiety may be substituted
15   with OH, -CONH$_2$, -CSNH$_2$, -CONHOH, -CSNHOH, -NHCHO, -NHCONH$_2$,
-NHCSNH$_2$, -NHSO$_2$NH$_2$ or -SO$_2$NH$_2$; or aryl, aryl(C$_{1-2}$)alkyl,
heterocyclyl, or heterocyclyl(C$_{1-2}$)alkyl which may optionally
be substituted in the aryl or heterocyclyl moieties with one,
two or three substituents selected independently from OH, F,
20   Cl, NH$_2$, CONH$_2$, NHCOH, and SO$_2$NH$_2$;

X$_1$ is -O-, -S- or -NH-;

R$_6$ is H or, when X$_1$ is NH, acyl, HOCNH-Val-Met-, HOCNH-Ile-
(S,S)-dioxo-methionyl- or HOCNH-Val-(pyran-4-on-2-yl)-ala-
nyl-;

25   or a salt thereof.

23. A novel compound of the general formula

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wherein

X is O, P, P(O), S, SO, SO_2, CH_2, C(OH)H, or a group NQ_{11}, wherein Q_{11} is H, OH, C_{1-24} acyl or C_{1-24} alkyl;

5   Z_{11} is a bond, O, CH_2, S, SO, SO_2, or a group NQ_{12}, wherein Q_{12} is H, C_{1-24} acyl or C_{1-24} alkyl;

R_{11} is H; C_{1-24} alkyl, C_{1-24} alkenyl, or C_{1-24} alkynyl, which may be substituted with one ore more substituents indepen-dently selected from -OH, -COOH, -F, -Cl, $-CONH_2$, $-CSNH_2$, 10   -CONHOH, -CSNHOH, -NHCHO, $-NHCONH_2$, $-NHCSNH_2$, $-NHSO_3NH2$ and $-SO_2NH_2$; acyl; or $-(CH_2CH_2O)_s$-H, wherein s = 1, 2, or 3;

or R_{11} is $CH=CH-(CH_2)_n$'$-Q_{13}$ or $-(CH_2)_n$'$-Q_{13}$, wherein Q_{13} is an aryl or a heteroaryl group substituted with OH, COOH, F, Cl, $-CONH_2$, $-CSNH_2$, -CONHOH, -CSNHOH, -NHCHO, $-NHCONH_2$, $-NHCSNH_2$, 15 $-NHSO_3NH2$ and $-SO_2NH_2$, and wherein n' ≥ 0;

R_{12} and R_{13} are independently OH, H, F, Cl, OW_{11}, or $O(CO)W_{11}$, wherein W_{11} is C_{1-24} alkyl, C_{1-24} alkenyl or C_{1-24} alkynyl, or an aryl or a heteroaryl group substituted with -OH, -COOH, -F, -Cl, $-CONH_2$, $-CSNH_2$, -CONHOH, -CSNHOH, -NHCHO, $-NHCONH_2$, 20 $-NHCSNH_2$, $-NHSO_3NH2$ and $-SO_2NH_2$;

Z_{12} is a bond, O, S, or CH_2;

R_{14} is $-(CH_2)_n$''$-Q_{14}$, wherein Q_{14} is an aryl group or a hete-roaryl group substituted with -OH, -COOH, -F, -Cl, $-CONH_2$, $-CSNH_2$, -CONHOH, -CSNHOH, -NHCHO, $-NHCONH_2$, $-NHCSNH_2$, 25 $-NHSO_3NH2$ and $-SO_2NH_2$, and wherein n'' = 0, 1, 2, or 3;

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Z_{13} is a bond, O, CH_2, S, SO, SO_2, $NQ_{14}Q_{15}$, wherein Q_{14} is H, C_{1-24} acyl or C_{1-24} alkyl, and Q_{15} is CO or $-C(O)W_{12}$, wherein W_{12} is O or NW_{13}, wherein W_{13} is H, OH, C_{1-24} acyl or C_{1-24} alkyl;

5   R_{15} is H; C_{1-24} alkyl, C_{1-24} alkenyl or C_{1-24} alkynyl, which alkyl, alkenyl and alkynyl may be substituted with one or more substituents independently selected from, -OH, -COOH, -F, -Cl, $-CONH_2$, $-CSNH_2$, -CONHOH, -CSNHOH, -NHCHO, $-NHCONH_2$, $-NHCSNH_2$, $-NHSO_3NH2$ and $-SO_2NH_2$; acyl or $-(CH_2CH_2O)_s$-H, where-
10   in s = 1, 2, or 3;

or R_{15} is $CH=CH-(CH_2)_{n'}-Q_{13}$, or $-(CH_2)_{n'}-Q_{13}$, wherein Q_3 is as defined above and wherein $n' \geq 0$;

or a salt thereof.

15   24. A compound according to claim 22 or 23, which causes a prevention, inhibition or enhancement of the binding of G1'-19'WT to *PapD*, and/or causes a prevention, inhibition or enhancement of the binding of MBP-G140' to *PapD*, and/or causes a prevention, inhibition or enhancement of the binding
20   of G125'-140' to *PapD* and/or causes a prevention, inhibition or enhancement of a pilus subunit to *PapD*.

25. A novel pyranoside or a salt thereof, which is selected from the group consisting of
Ethyl 2,3-O-Dibenzoyl-4-O-benzyl-1-thio-β-D-glucohexopyrano-
25   side;
Ethyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-1-thio-β-D-gluco-
hexopyranoside;
Methylglycolyl 6-O-acetyl-2,3-O-dibenzoyl-4-O-benzyl-β-D-glu-
cohexopyranoside;
30   2-(Hydroxy)ethyl 4-O-benzyl-β-D-glucopyranoside;
Sodium glycolyl 4-O-benzyl-β-D-glucohexopyranoside;
Methyl 2-O-ethyl-4,6-O-(4'-methoxy)phenylmethylene-α-D-manno-
hexopyranoside;

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Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,6-O-(4'-me-
thoxy)phenylmethylene-α-D-mannohexopyranoside;

Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-methoxy)-
benzyl-α-D-mannohexopyranoside;

5   methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-6-O-(4'-methoxy)-
benzyl-α-D-mannohexopyranoside;

Methyl 2-O-ethyl-3-O-dimethyl-t-butylsilyl-4,-O-(4'-methoxy)-
benzyl-6(S)-phenyl-α-D-mannohexopyranoside;

Methyl 2,3-anhydro-4,6-O-p-methoxybenzylidene-α-D-mannopy-
10   ranoside;

Methyl 3-azido-4,6-O-p-methoxybenzylidene-α-D-altropyrano-
side;

Methyl 3-azido-2-O-ethyl-4,6-O-p-methoxybenzylidene-α-D-
altropyranoside;

15   Methyl 3-azido-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-α-D-
altro-pyranosid;

Methyl 3-azido-6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxy-
benzyl-α-D-altropyranoside;

Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
20   sulfamino-α-D-altropyranoside sodium salt;

Methyl 6-O-benzoyl-3-deoxy-2-O-ethyl-3-sulfamino-α-D-altropy-
ranoside ammonium salt;

Methyl 3-azido-6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxy-
benzyl-α-D-altropyranoside;

25   Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
sulfamino-α-D-altropyranoside sodium salt;

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-3-sulfamino-α-D-altro-
pyranoside ammonium salt;

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-4-O-p-methoxybenzyl-3-
30   tbutyloxamido-α-D-altropyranoside;

Methyl 6-O-pivaloyl-3-deoxy-2-O-ethyl-3-oxamido-α-D-altropy-
ranoside ammonium salt;

Methyl 3-azido-6-O-pyrrol-3'-ylcarboxyl-3-deoxy-2-O-ethyl-4-
O-p-methoxybenzyl-α-D-altropyranoside; and

35   Methyl 6-O-pyrrol-3'-ylcarboxyl-3-deoxy-2-O-ethyl-3-sulfami-
no-α-D-altropyranoside ammonium salt.

26. A method for identifying a binding site in a periplasmic molecular chaperone or an analogue thereof, comprising

co-crystallizing the periplasmic molecular chaperone or an analogue thereof with a ligand binding to the periplasmic
5   molecular chaperone or the analogue thereof,

resolving the three-dimensional structure of the chaperone/ligand interaction, thereby resolving the three-dimensional structure of the periplasmic molecular chaperone or the analogue thereof when binding to the ligand,

10   determining the site-point(s) involved in the intermolecular interaction between the periplasmic molecular chaperone or the analogue thereof and the ligand, and

identifying the thus determined site-point(s) of the periplasmic molecular chaperone or the analogue thereof as a
15   binding site in the periplasmic molecular chaperone or the analogue thereof.

27. A pharmaceutical composition, comprising, as an active compound, a substance capable of interacting with at least one type of periplasmic molecular chaperone which binds pilus
20   subunits during transport of these pilus subunits through the periplasmic space and/or during the process of assembly of the intact pilus, in such a manner that binding of pilus subunits to the periplasmic molecular chaperone is prevented, inhibited or enhanced, in combination with at least one
25 pharmaceutically acceptable carrier or excipient.

28. A pharmaceutical composition, comprising, as an active compound, a substance according to any of claims 22-25 or a substance identified according to the method in any of claims 8-21, in combination with at least one pharmaceutically
30 acceptable carrier or excipient.

29. A pharmaceutical composition according to claim 28, which further comprises at least one additional pharmaceutical substance.

30. A pharmaceutical composition according to claim 29,
5 wherein the at least one additional pharmaceutical substance is an antibacterial agent selected from the group consisting of penicillins, cephalosporins, aminoglycosides, sulfonamides, tetracyclines, chloramphenicol, polymyxins, antimycobacterial drugs and urinary antiseptics.

10 31. A substance for use as a pharmaceutical, the substance being capable of interacting with at least one type of periplasmic molecular chaperone which binds pilus subunits during transport of these pilus subunits through the periplasmic space and/or during the process of assembly of the intact
15 pilus, in such a manner that binding of pilus subunits to the periplasmic molecular chaperone is prevented, inhibited or enhanced.

32. A substance according to claim 31, wherein the binding site is a binding site to which the carboxyl terminal part of
20 a pilus subunit is capable of binding, and which comprises site points substantially identical to the invariant residues Arg-8 and Lys-112 in PapD, and a polypeptide fragment which is capable of interacting with a β-strand of the carboxyl terminal part of the pilus subunit, thereby stabilizing the
25 binding of said subunit at the Arg-8 and Lys-112 site points of the binding site.

33. A substance according to claim 32 which causes a prevention, inhibition or enhancement of the binding of G1'-19'WT to PapD, and/or causes a prevention, inhibition or enhance-
30 ment of the binding of MBP-G140' to PapD, and/or causes a prevention, inhibition or enhancement of the binding of G125'-140' to PapD and/or causes a prevention, inhibition or enhancement of a pilus subunit to PapD.

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34. A substance according to any of claims 31-33 which has been identified according to the method as claimed in any of claims 8-21 or which is a compound according to any of claims 22-25.

5   35. The use of a substance which is capable of interacting with at least one type of periplasmic molecular chaperone which binds pilus subunits during transport of these pilus subunits through the periplasmic space and/or during the process of assembly of the intact pilus, in such a manner 10 that binding of pilus subunits to the periplasmic molecular chaperone is prevented, inhibited or enhanced, for the preparation of a pharmaceutical composition for the treatment and/or prophylaxis of bacterial infection.

36. The use according to claim 35, wherein the binding site 15 is a binding site to which the carboxyl terminal part of a pilus subunit is capable of binding, and which comprises site points substantially identical to the invariant residues Arg-8 and Lys-112 in PapD, and a polypeptide fragment which is capable of interacting with a β-strand of the carboxyl 20 terminal part of the pilus subunit, thereby stabilizing the binding of said subunit at the Arg-8 and Lys-112 site points of the binding site.

37. The use according to claim 35 or 36, wherein the substance has been identified according to the method as claimed 25 in any of claims 8-21 or wherein the substance is a compound according to any of claims 22-25.

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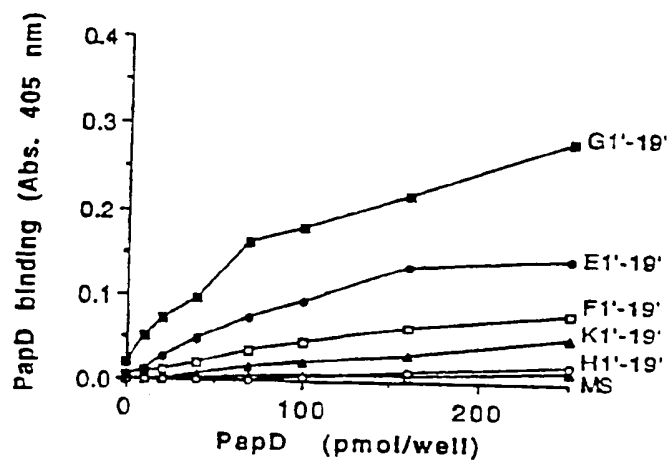
Fig. 1A
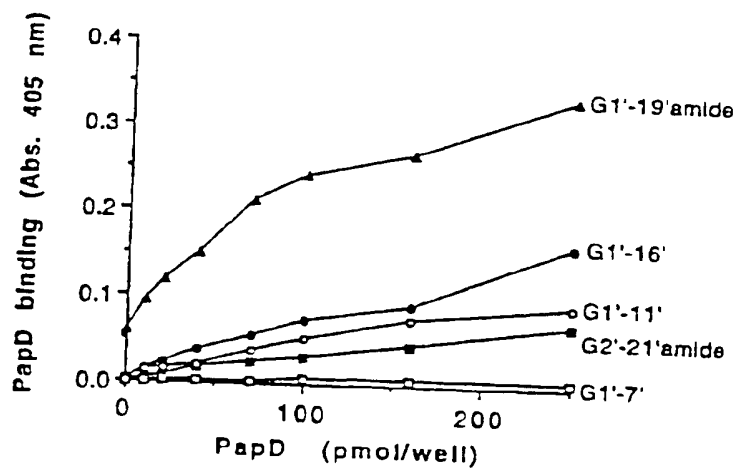
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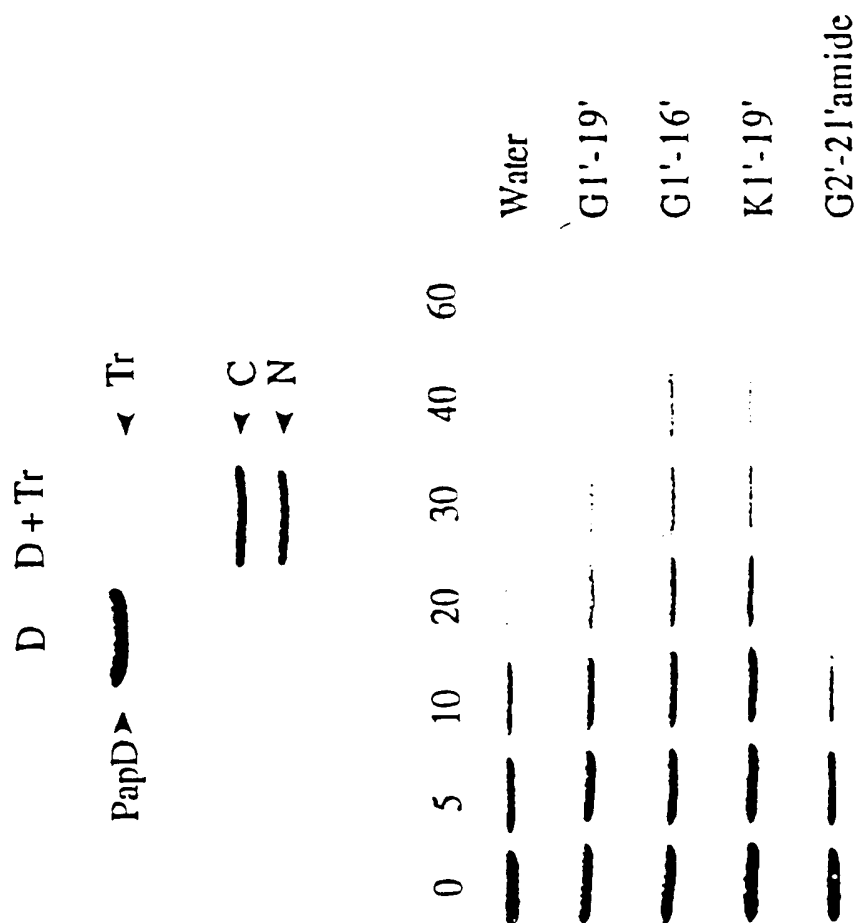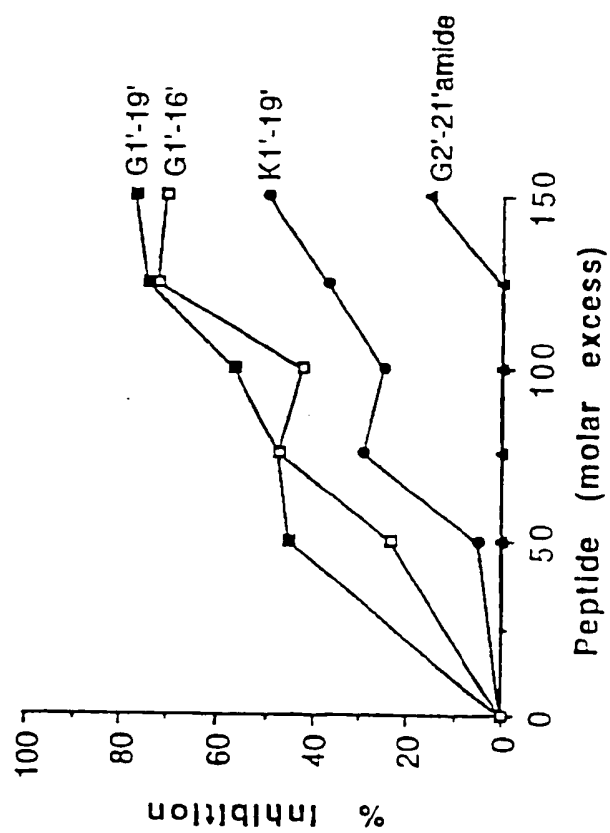
Fig. 2A

Fig. 2B

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

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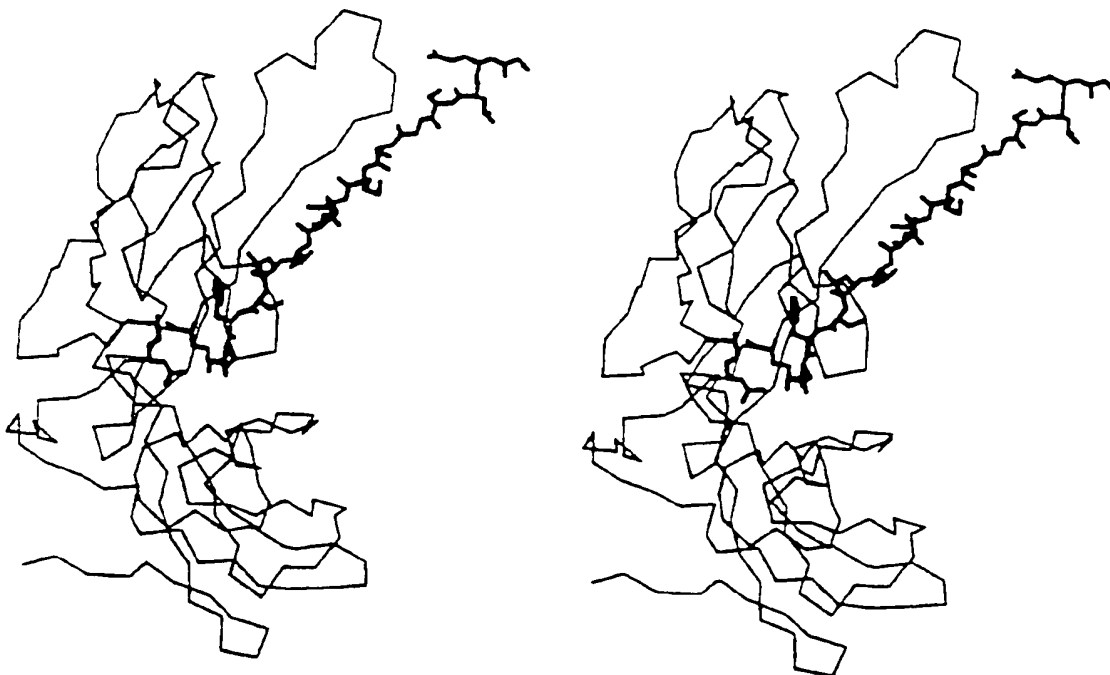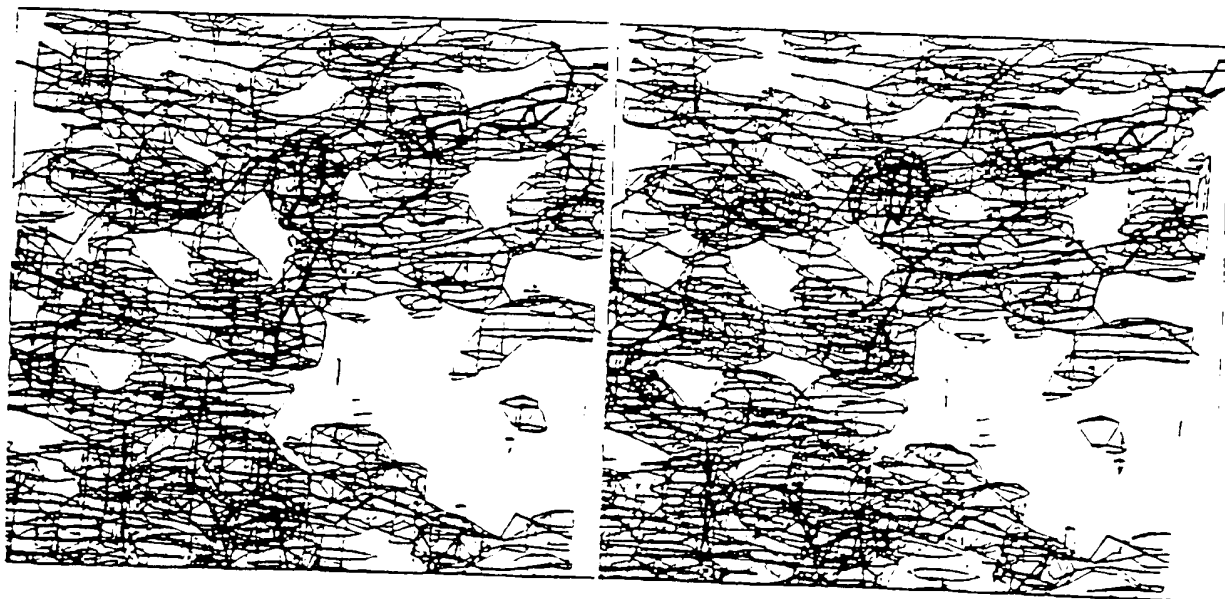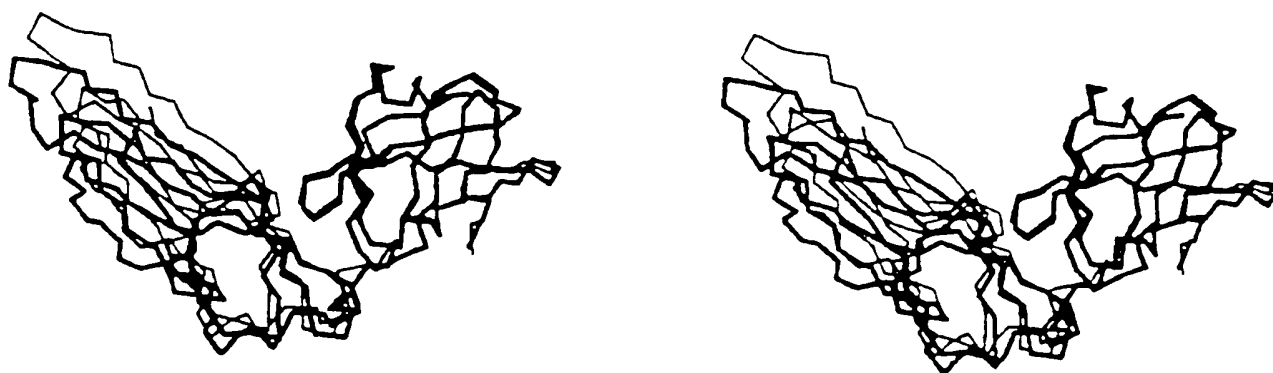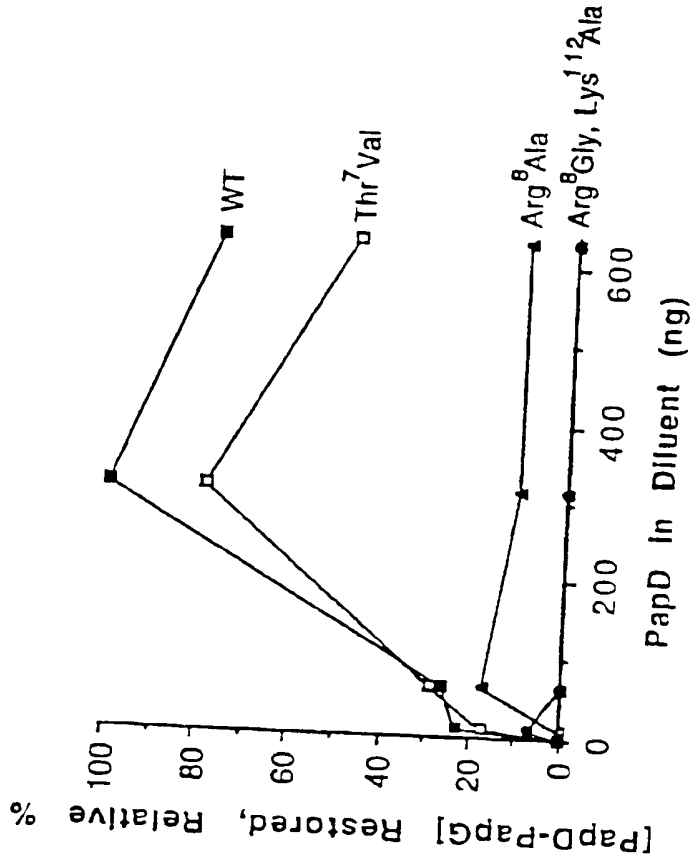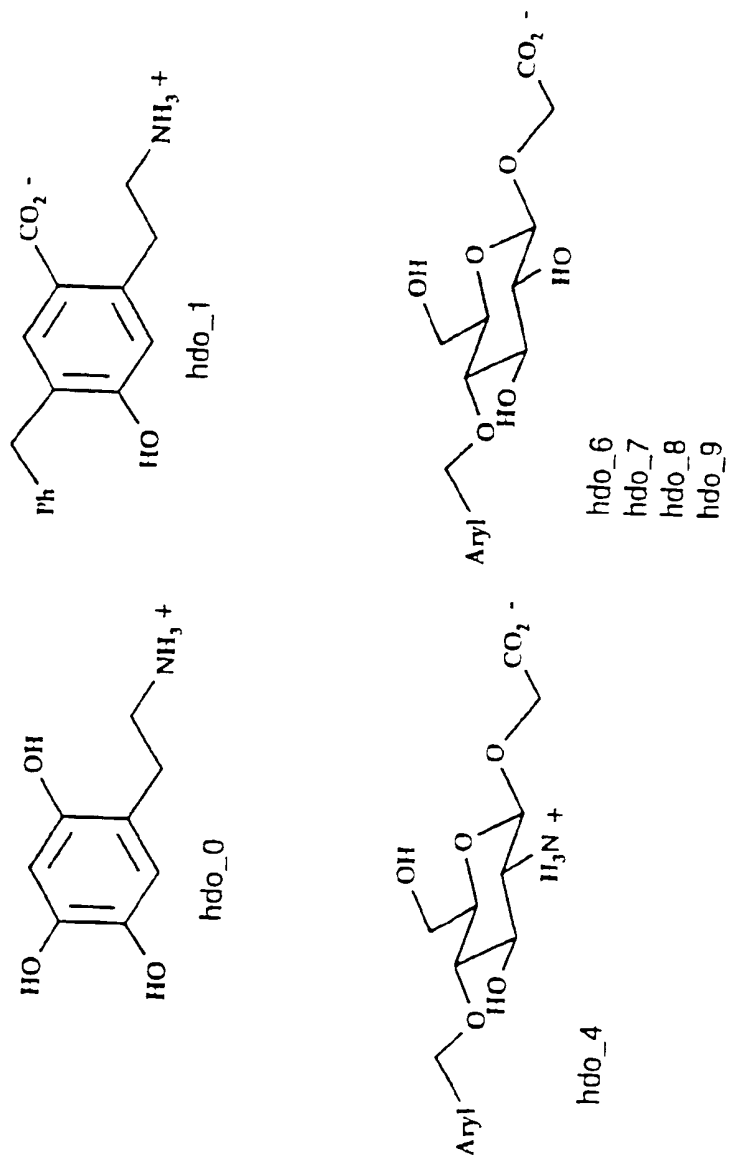
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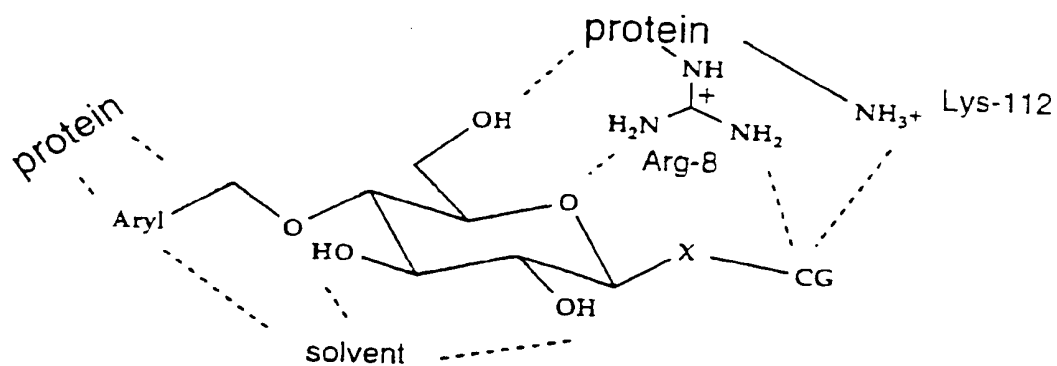
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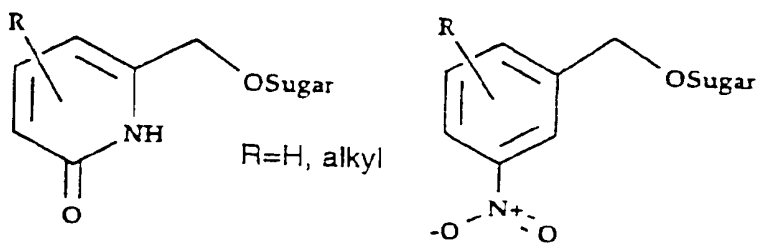
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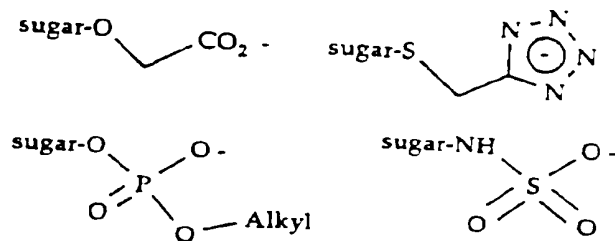
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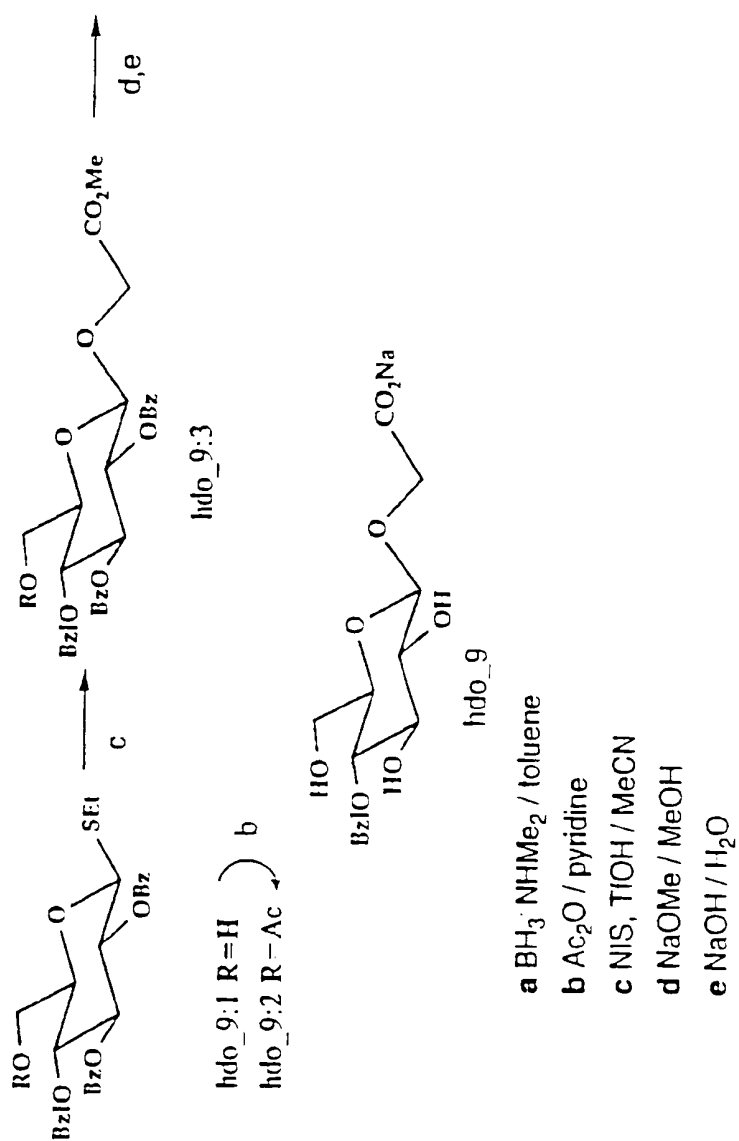
Fig. 10

10/24
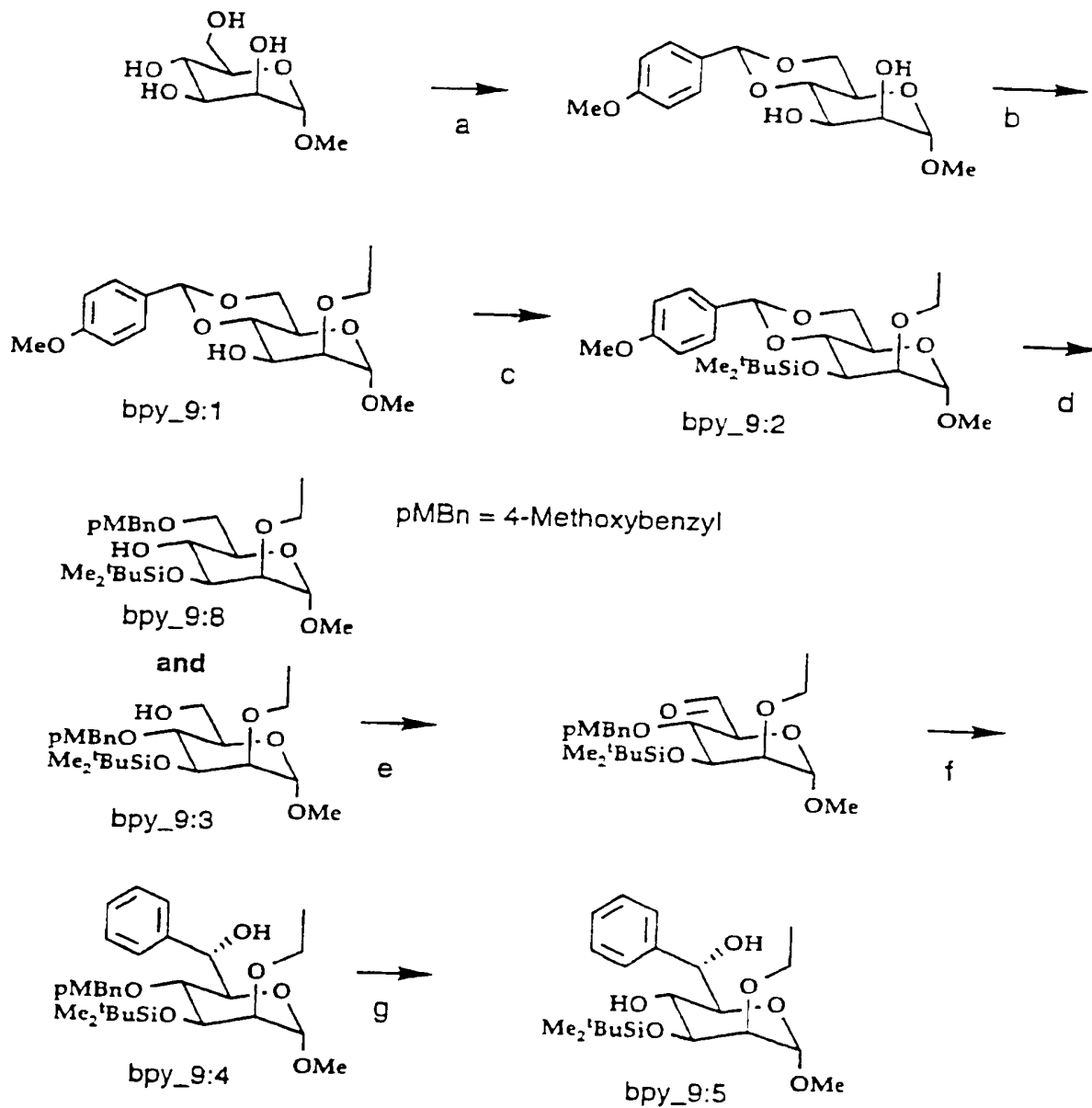
a p-MeOPhCH(OMe)₂,TsOH / MeCN
b EtI, NaOH, NBu₄HSO₄ / CH₂Cl₂, H₂O
c 'BuMe₂SiOTf, Et₃N / CH₂Cl₂ or 'BuMe₂SiCl / pyridine
d NaCNBH₃, Me₃SiCl / MeCN
   or NaCNBH₃, HCl(Et₂O) / Et₂O
e DMSO, P₂O₅, Et₃N / THF
f PhMgCl / THF
g CAN / MeCN, H₂O

Fig. 11

Fig. 12

h   Ac$_2$O / pyridine

l   NBu$_4$F 3H$_2$O / MeCN

j   (CF$_3$SO$_2$)$_2$O, / CH$_2$Cl$_2$, pyridine

k   NaN$_3$ / DMF

l   H$_2$S / pyridine

m   SO$_3$ / pyridine

n   NaOMe / MeOH
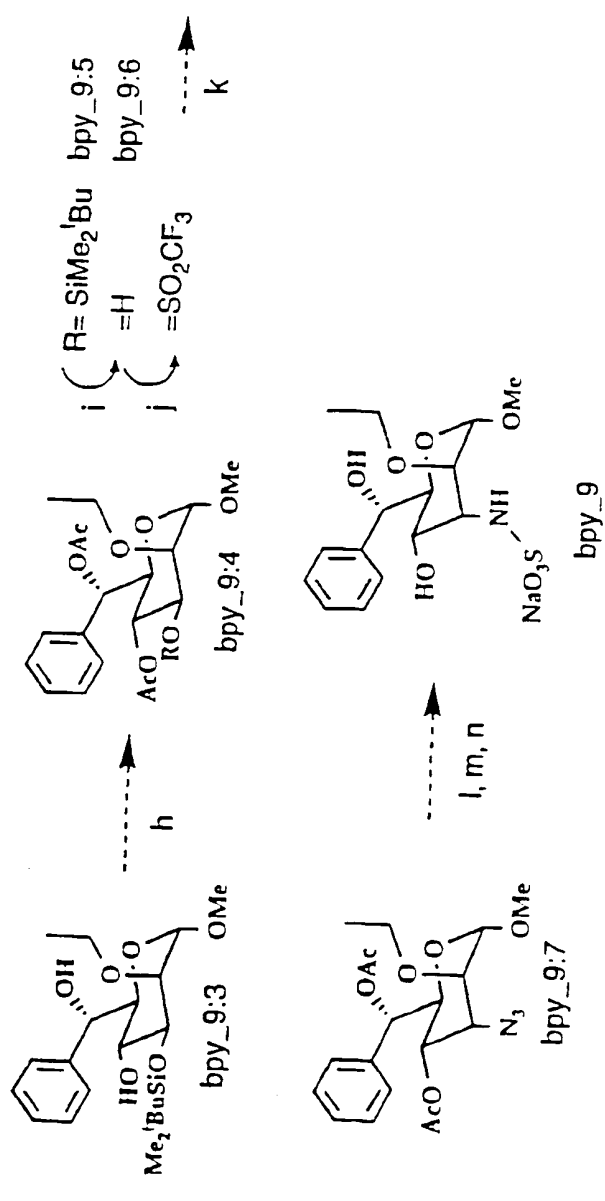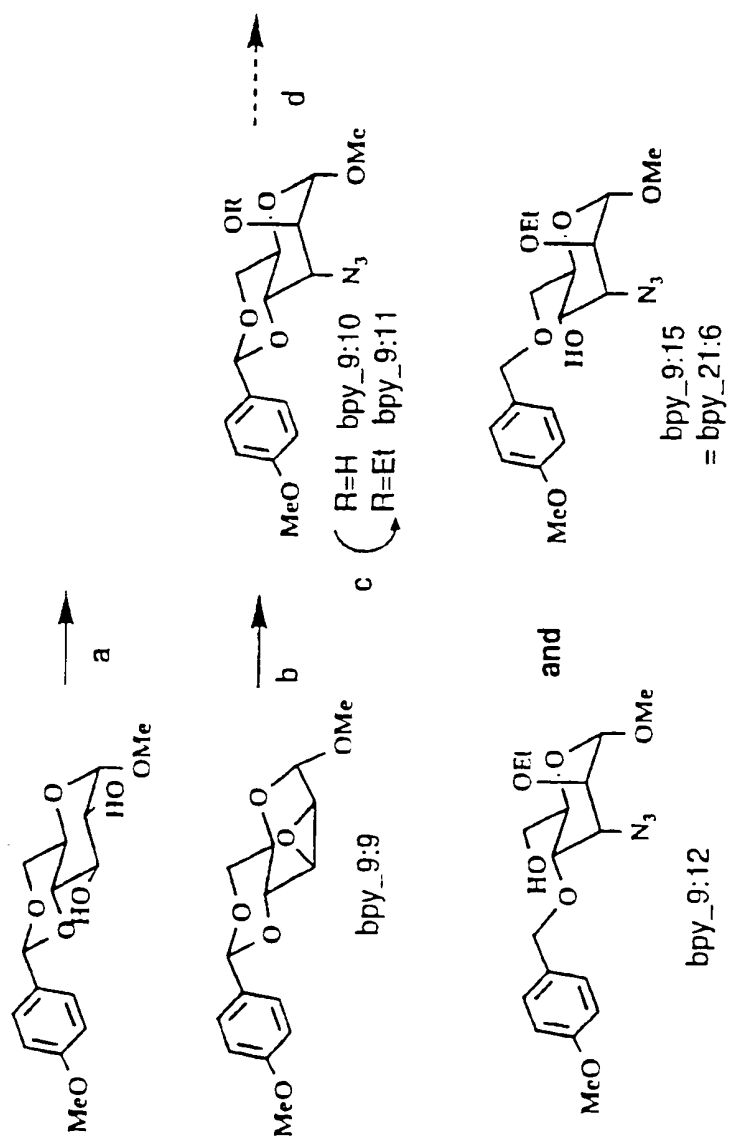
Fig. 13

a Imidazolyl tosylamide NaH / THF, rt, 66%
b NaN$_3$, NH$_4$Cl / EtOH, H$_2$O, reflux, 72%
c EtI, NaH / DMF or alkyl halide, NaH / THF
d NaCNBH$_3$, Me$_3$SiCl / MeCN

e Swem oxidation
f PhMgCl / THF
g CAN / MeCN, H₂O
h H₂S / pyridine
i SO₃ / pyridine
j AcOH / H₂O
k NaOH / H₂O

Fig. 14

Fig. 15

15/24

bpy_21:6

i H₂S / pyridine
j SO₃ / pyridine
k AcOH / H₂O
l NaOH / H₂O

bpy_21

Fig. 16

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Fig. 17

Replacement series
(Data from 7 experiments)

% Inhibition

G1'-8'   G1'-8'S1   S2   A3   S4   S5   S6   A7   S8

Fig. 18

Deletion series

(Data from 6/7 experiments)

Fig. 19

19/24

Fig. 21A

Fig. 21B

Fig. 21C

Fig. 21D

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MW
(kDa) 1   2   3   4

21-

◄PapA

14-

Fig. 22A

MW
(kDa) 1·   2   3   4

30-

◄PapG

◄PapD

Fig. 22B

MW
(kDa) 1   2   3   4

21-

◄PapK
◄PapE
◄PapF

14-

Fig. 22C

22/24

Fig. 23A

MBP and MBP/G protein concentration (pmol/well)

23/24

PapDG complex

PapD

PapD antisera

PapG antisera

24/24